Affinity Biosensor Interface Engineering for
Real-Time Biohazard Monitoring

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Declaration

I declare that the work in this thesis is my own.

Work of others has been referenced or attributed accordingly.

Neal A. E. Hopkins

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To Henry and Rosie - anything is possible!
Abstract

Our environments abound with biological materials which can impact upon our health. The consequence of our exposure can range from mild irritation to acute and life threatening infection. To defend effectively against exposure to this spectrum of biohazards detection systems must precisely interrogate complex heterogeneous mixtures of biological entities sampled from the environment. Such a system must respond instantaneously and alert those at risk of exposure so that appropriate action may be taken.

In principle, affinity biosensor technologies (e.g. surface plasmon resonance sensors) may be used within a detection system to continuously interrogate an aqueous sample (e.g. continuously delivered by an air sampler). The specific benefit of affinity biosensors is that, at the point of analysis, they are reagentless. This permits simple and automated operation without need of complex fluidics or mandrolic processing and, most importantly of all, on-line and near real-time analysis. All that is required to exploit these technologies is an affinity sensing interface which binds only the materials of concern and rejects any and all other materials. The concept is simple, but this belies the technical complexity of its realisation.

Recognition elements (e.g. antibodies) are broadly available as binding ligands for application in affinity sensors. However, their integration and performance within such technologies, facilitated by other necessary enabling components (e.g. hydrated polymers), presents a long standing and multidisciplinary technical challenge. Current technologies are prone to false indications making them unreliable. For example; spontaneous non-specific biological adsorption compromises binding specificity yielding false positive results, and variable integration of recognition elements (e.g. random orientation) may compromise sensitivity yielding false negative results.

Theoretical models suggest the necessary functionality required of affinity biosensor interfaces is a credible possibility (e.g. those built upon the polymer brush model described by de Gennes in 1980 [1]). However, even the most recent iterations of such models are not predictive of interfacial biological processes as the molecular behaviour of the solvent (i.e. water) is excluded (principally as it is both ill-defined and computationally challenging). There is, therefore, a need for further empirical exploration of such biological interfaces to understand how materials interact within such systems and how to fabricate them.

The work herein has systematically, and at the molecular scale, engineered different components of the affinity biosensor interface. The aim has been to develop an interfacial system to enable real-time environmental affinity biosensing to become a practical reality, i.e. to resist all non-specific biological adsorption, and reproducibly integrate biological recognition elements. Surface plasmon resonance has been used throughout as a representative affinity biosensor technology. This class of affinity biosensors
are sensitive and the transduction mechanism does not physically or chemically perturb the sensing interface (e.g. by introduction of mechanical or electrochemical processes) and is, therefore, unlikely to change the behaviour of the interfacial system which is being interrogated. This should enable facile data interpretation in pursuit of new design rules for biosensor interface engineering. Specific objectives were as follows;

- To engineer recombinant antibodies to simultaneously enable both site-specific chemical modification of the product, and facile manufacture within an industrial microbial expression system.
- To engineer thin-film polymer coatings to passivate a SPR interface so that it may resist all detectable non-specific adsorption from complex and heterogeneous biological mixtures.
- To demonstrate specific biological detection within complex and heterogeneous biological mixtures through the immobilisation of site-specifically engineered recombinant antibodies upon a non-specific adsorption resistant SPR interface.

These objectives have been partially met. The principle outcomes are as follows;

- A novel protein engineering tool has been developed and, where *E. coli* periplasmic expression is used to manufacture recombinant antibodies, a new novel tag may be universally applied which does not reduce soluble yield. Once purified the intrinsic, labile chemistry within the tag may be selectively activated via the reduction of an intrinsic disulphide bond to yield two free thiols for subsequent manipulation.
- A novel affinity biosensor interface has been developed. All covalent interfacial construction methods assessed (e.g. ATRP or SAM deposition methods of zwitterionic or oligoethylene glycol functionalities) yielded thin-films which adsorbed protein non-specifically. These were discarded and a non-covalent, self-organising interface was developed. Adsorption from undiluted animal serum was found to be undetectable on this novel affinity interface. The properties of the self-organising molecular components are; they all present pendant oligoethylene glycol of between 7 and 10 repeats, as surfactants they reduce the surface tension of water to 30 mN.m\(^{-1}\) or below, they have hydrophobic functionalities which match the underlying hydrophobic interface, or they are more hydrophobic than the interface (though the surface must also be hydrophobic).
- The covalent integration of recognition elements was successfully demonstrated by the insertion of polyethylene ether tethers within the non-covalent self-organising coating. However, once again, these surfaces were found to foul non-selectively. The recurrence of non-selective adsorption is thought to be attributable to the physical behaviour of the tether where localised chain entropy is reduced. As far as the author is aware, this is the first observation of protein fouling that may be
directly attributed to hydrated polyethylene ether which has not been driven by exogenous mechanical or chemical forces.

The tools and understanding demonstrated through SPR sensing are anticipated to find broad utility across affinity sensors. This work presents a tangible start point to study the behaviour of complex soft matter assemblies, and to build more functional self-organising interfacial molecular systems.

Prospective future work:

- The biosensor interface must be engineered as a concerted soft matter system. The integration of recognition elements within the non-covalent, self-organising coating must be undertaken in a way that sustains the entropy of the molecular tethers or, alternatively, completely shrouds them to sterically preclude fouling. However, considering the need to covalently immobilise the recognition element, and therefore reduce entropy at least at this specific point, it is possible that non-specific adsorption is simply a fundamental thermodynamic inevitability. This may therefore defeat the simple concept of real-time affinity biosensing, at least through mass sensing modalities.

- As a broad enabler of affinity biosensor platforms, the self-organising building blocks defined herein should be amenable to systemic molecular engineering to further integrate properties to suit varied transduction principles, e.g. redox activity. The opportunity to augment optical sensors with electrochemical functionality should enable confirmatory analytical tests to be undertaken upon the same sensor interface. The consequence of any residual non-specific adsorption may then be negated.

- The self-organising system may also be amenable to phase partitioning to spatially direct specific molecular species in the system to predefined locations, e.g. upon a hydrophobically patterned surface, or solvated components such as nanoparticles. In so doing it may be possible to further develop the self-organising concept to explore interfacial assemblies and molecular electronics which may obviate the need of the optical sensing modality (leading further to miniaturisation etc.).

- Beyond environmental biosensing, the interface may find application in basic research studying biological interactions in complex and congested molecular environments. This may be applied to generate insights into complex molecular processes such as; interaction dynamics of intrinsically disordered proteins, behaviour of DNA-protein interactions within the nucleoplasm, or amyloid formation in varied physiological environments. Quantitative insight into such biological soft matter systems may be a broad enabler of computational modelling to benefit bioengineering disciplines such as synthetic biology and medical therapeutics.
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Affinity Biosensor Interface Engineering for

Rapid Biohazard Detection

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Abbreviations

ABTS 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt
AFM Atomic force microscopy
ARGET ATRP Activators regenerated by electron transfer ATRP
ATRP Atom transfer radical polymerisation
BLI Biolayer interferometry
BSA Bovine Serum Albumin
CCD Charge-coupled device
Da Dalton
dAb Domain antibody (IgNARv or V\textsubscript{H})
DMAP 4-(dimethyl-amino)pyridine
DMF Dimethyl formamide
DNA Deoxyribonucleic acid
DPA Dipicolinic acid
DSC Disuccinimidyl carbonate
DTT Dithiothreitol
E. coli *Escherichia coli*
EDC 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EIS Electrochemical impedance spectroscopy
ELISA Enzyme Linked Immunosorbent Assay
HEL Hen egg lysozyme
IAEDANS 5-[2-[(2-iodo-1-oxoethyl)amino]ethylamino]-1-naphthalenesulfonic acid
IDP Intrinsically Disordered Protein
Ig Immunoglobulin
IgG Immunoglobulin Gamma
IgNARv Shark single domain antibody
MA Methacrylate
MALDI-TOF Matrix-assisted laser desorption and/or ionization time of flight mass spectrometry
NHS N-hydroxysuccinimide
NMR Nuclear Magnetic Resonance
OEG Oligoethylene glycol
OP Organophosphorus
OWLS Optical waveguide lightmode spectroscopy
PBST Phosphate Buffered Saline Tween
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulphide isomerase</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol (hydroxyl terminated polyethylene oxide)</td>
</tr>
<tr>
<td>PEO</td>
<td>Polyethylene oxide</td>
</tr>
<tr>
<td>pKa</td>
<td>Acid dissociation constant</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz Crystal Microbalance</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>SA</td>
<td>Streptavidin</td>
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<td>SAW</td>
<td>Surface acoustic wave</td>
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<td>SAM</td>
<td>Self Assembled Monolayer</td>
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<td>scFv</td>
<td>Single chain fragment variable antibody</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>SPR</td>
<td>Surface Plasmon Resonance</td>
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<td>TAE</td>
<td>Tris acetate EDTA</td>
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<td>Tris(2-carboxyethyl)phosphine</td>
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1 Introduction

1.1 Defence Against Environmental Biohazards

1.1.1 The Nature of Biohazards and Human Sensitivity

In popular culture biohazards have become synonymous with biological warfare and bioterrorism\(^1\). The use of acutely biohazardous materials as weapons of war and instruments of terror has unfortunately been a consistent theme in human history; from the siege of Caffa in 1346 [2] to the “anthrax letters” of 2001 [3]. In such cases biohazards have been deliberately introduced into human environments in order to harm those present. In this context a biohazard may be defined as a biologically derived material which is inherently toxic to humans or is itself the causative agent of human disease.

Today, the development and use of biohazards as weapons is prohibited by the Biological and Toxin Weapons Convention [4]. Originally championed by Britain in 1972, this convention has been broadly adopted with 180 states party to it (as of January 2018 [4]). Prior to this date, historic activities within many industrialised nations explored acutely biohazardous materials as putative biological weapons [5], for example; toxins (e.g. botulinum toxin [6]), viruses (e.g. Venezuelan equine encephalitis virus [7]) and bacteria (e.g. *Bacillus anthracis* [8]).

While acute biohazards have become notorious by military connotation, our environments abound with other biological materials which can impact upon human health. The consequence of which can range from mild irritation to chronic infection, for example; pollen allergens (e.g. *Cryptomeria japonica* [9]), mycotoxins (e.g. aflatoxin [10]) and fungal spores (e.g. *Aspergillus fumigatus* [11]). A definition of environmental biohazards must, therefore, be broad to encompass any biologically derived material known to have a detrimental impact upon human health. A vast range of materials may therefore be considered to be environmental biohazards.

Such a huge range of potentially harmful materials defies precise physical or chemical definition. In physical terms biological entities vary greatly in size from nanometres (for molecular toxins, e.g. aflatoxin) to micrometres (for bacterial cells, e.g. *Bacillus anthracis*). From a chemical perspective, biological entities are relatively homogenous, being composed of common atomic species (principally; carbon, hydrogen, oxygen, nitrogen and sulphur) assembled into similar molecular species (e.g. lipids, amino acids and

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\(^1\)“Biohazard” in Wikipedia correlates with “Biological Warfare” and “Bioterrorism” https://en.wikipedia.org/wiki/Biohazard
nucleic acids). Therefore, in general terms, biological materials are physically heterogeneous yet chemically homogenous. To compound matters, seasonal and diurnal variations in both frequency and composition can also be profound [12].

However, while their chemical components may be common their composites can be unique. For example, polymers of common molecular components (e.g. polysaccharides, polypeptides and polynucleotides) may contain unique sequences occurring only in specific biological entities. Only through analysis of these composite biopolymers is it possible to directly differentiate between biological materials.

We may be passively exposed to environmental biohazards in three ways; skin contact, ingestion or inhalation. Our sensitivity to exposure is different in each case. The barrier presented by the skin is surprisingly effective in blocking the passage of biohazards into our bodies and so the hazard posed, even by acute biohazards such as *Bacillus anthracis*, is significantly reduced. The gut is more sensitive to exposure, but the extreme pH within the stomach, and commensal gut flora, can mitigate biohazards and reduce our sensitivity considerably, even to acutely toxic materials such as ricin toxin [13]. We are most sensitive to exposure via inhalation due to the critical function of the lungs and a deficit of physiological protective mechanisms within this organ to directly mitigate any biohazards encountered.

Humans are unable to directly sense the molecular character of biopolymers as they are encountered. Therefore, should a biohazard pervade our environment, we are oblivious to its presence and cannot respond appropriately to reduce our exposure. However, humans (and other higher eukaryotic organisms) have evolved sophisticated machinery (i.e. the humoral immune response) through which to identify differences between human and non-human biopolymers (e.g. three-dimensional protein or lipopolysaccharide motifs)\(^2\). However, it takes days to develop this sophisticated, “antibody” mediated, molecular level defence. In the same timeframe, disease progression from acute causative agents (e.g. *Yersinia pestis*) can overwhelm the less sophisticated innate immune mechanisms [15]. However, should we survive our first encounter with a biohazard, the humoral immune system is most beneficial as the newly acquired targeted immunity is sustained through life (hence the success of vaccination in disease control). However, for our first exposure, and for a period of days after, we are without the specific

\(^2\) The humoral immune response serves to protect from acute hazards but the system can also sensitise us to benign materials which we may be variably exposed to, such as plant pollens. These materials are not acutely hazardous but they are still of consequence to human health and wellbeing. (14. Schmid-Grendelmeier, P., et al., Native Art v 1 and recombinant Art v 1 are able to induce humoral and T cell-mediated in vitro and in vivo responses in mugwort allergy. Journal of Allergy and Clinical Immunology, 2003. 111(6): p. 1328-1336.)
molecular level defence required, during which time biohazards may act upon our bodies largely unchecked.

1.1.2 Detection Systems for Defence Against Environmental Biohazards

Key requirements of a detection system to defend against environmental biohazards are;

- **Speed**: Bioanalysis must be completed and reported quickly to enable actions to be taken to avoid or minimise our exposure to any detected hazard.
- **Specificity**: The analysis must be specific and not falsely indicate the presence of hazards where none are present.
- **Sensitivity**: If sensitivity is not sufficient, false negative indications may result and the system will not alert those to the biohazard present.
- **Stability**: If components within the detection system are labile then the maintenance required to sustain performance may be considerable, either in terms of climatic control or replacement of consumables. This may increase the cost of running such a system.
- **Simplicity**: If the system is complex this may necessitate mandric processing or maintenance to sustain operation/performance. This may also increase the cost of running such a system.

Considering the nature of biohazards, and our sensitivity to them, a detection system should ideally work in real-time and interrogate biopolymers in the aerosol phase. Such a system may find broad utility, from the management of industrial facilities (e.g. poultry farms [16] and compost sites [11]) to defence against bioterrorism [3].

Simple physical and chemical properties can be probed in real-time within the aerosol phase (e.g. light scattering and fluorescence of aerosol particles [17]). These methods are quick but attributing any observations from such amenable analytical techniques to any specific biohazardous entity is unreliable due to the high variance of physical parameters and the general occurrence of the simple chemical signatures probed. For example, dipicolinic acid (DPA) is a chemical signature associated with bacterial endospores, such as those produced by *Bacillus anthracis* [18]. DPA may be detectable in real-time via bioaerosol mass spectrometry [19] but DPA occurs within hazardous and non-hazardous materials alike being common within bacterial endospores [20]. DPA monitoring may find utility as an indicator that the abundance of endospores may have increased within an environment but such an observation may be attributable to changes in the prevailing wind [21] rather than the onset of a bioterror attack. Greater specificity is required. Within a detection system such sensors may find utility as triggers for more sophisticated analytical techniques but as independent sensors they do not provide compelling information. Biohazards need to be specifically identified.
By interrogating the composite biopolymers of biomaterials more specific information may be derived. All technologies thus capable require the biological entities to be in the liquid phase. Therefore, a bioaerosol collection device (e.g. impinger or cyclone [22]) must be employed to actively partition the bioaerosol into a liquid phase.

Traditional methods (developed since the time of Pasteur [23]) successfully allow the identification of biological microorganisms (including biohazards) through empirical assessment of gross physical manifestations (which may be indirectly attributed to physical properties of composite biopolymers and their combinations). Traditional biological screening methods require extensive time, highly trained people and a laboratory environment with associated equipment, for example; the mouse lethality assay for botulinum toxin [24] and cytopathic microscopy for VEE virus [25]. Though these examples may appear exotic more broadly applied microbiological screening techniques are similarly constrained, e.g. culturing bacteria using selective media and assessing the morphology of subsequent bacterial colonies. So, while traditional methods can identify biohazards, they are difficult to implement and slow to complete, especially when a wide range of biohazards are to be simultaneously considered.

Clinical applications are a significant driver of technological development where bioanalysis is concerned. This has spurred the augmentation of traditional methods, e.g. to increase throughput via automation though MALDI-TOF mass spectrometry, or to reduce subjectivity of colony morphology assessment [26]. While such augmentation has been clinically useful, fundamentally, any method requiring the controlled culture of microbiological entities will not be suitable for responsive biohazard monitoring due to the time and complexity required.

More recent technological developments allow direct sequencing of biopolymers in order to derive information more quickly. All biopolymers are information rich but directly determining their primary sequences still takes time due to the varied complexities of each method. Relative to polysaccharide sequencing (e.g. very challenging but possible via mass spectrometry [27]), the sequencing of polynucleic acids (e.g. via “next-generation” DNA sequencers [28]) and polyamino acids (e.g. via tryptic peptide mass spectrometry [29]) are relatively trivial. Detected primary biopolymer sequences may be analysed using bioinformatics to specifically identify biological entities (i.e. statistical comparative analysis of detected sequences against libraries of archived data from all reported biological entities [30]). The direct sequencing of biopolymers can provide a high level of analytical specificity. However, these methods generally take time to both evolve the data and statistically analyse it. In addition, specific issues erode the benefits, e.g. the frequency of errors within derived sequences (e.g. for DNA sequencing [31]) and blind spots where no sequence coverage is returned (e.g. for certain methods of MS peptide mapping [32]). The consequence of inconsistent and error prone sequence data undermines the value in deriving it.
False positives and negatives may result as key features within primary sequences may be attributable to single points within the polymers (e.g. single point polymorphisms within *Bacillus anthracis* Ames strain [33]). In addition, the processing required to derive this information can be intensive of both time and expertise.

DNA sequencing technology is rapidly evolving and while the speed and automation of these platforms increases the compromise is the fidelity of the derived information. In some applications the DNA of interest will occur with high frequency (e.g. diagnostic tissue typing), and redundancy in parallel sequencing data allows sequence errors to be identified bioinformatically. However, if DNA signatures are present in low frequency, and within a background, then any introduced error cannot be excised and the value in the data is eroded. As with the simple detection systems which may interrogate bioaerosols directly, nanopore DNA sequencers could be used to trigger more specific analysis (e.g. PCR) and thus system complexity increases. It is probable that the fidelity of these sensors will improve and it is likely a matter of time before they return high fidelity and lengthy sequence data, but as extensive sequence databases propagate, with varying levels of annotation, real-time data interpretation may well prove more of a challenge.

An alternative approach to direct biopolymer sequencing is to probe affinity interactions with cognate binding partners. The shape/structure of proteins is predetermined by their composite polyamino acid sequences. It is possible to detect these specific physicochemical characteristics by affinity interactions and thus infer the presence of the specifically associated biopolymers. Indeed, it is these material signatures which are targeted by the humoral immune system and, as already mentioned in section 1.1.1, this system dynamically evolves antibody molecules as affinity ligands with which to label biohazards for assault by cellular agents of the immune system.

Since the discovery of antibody mediated phenomena by Kitasato in 1890 [34] these remarkable affinity ligands have been the subject of colossal research and investment. Technologies are now available which allow *in vitro* development of antibody affinity ligands to almost anything. Through affinity interaction analysis these materials may be used to rapidly interrogate the physicochemical nature of molecular analytes to determine whether any specific motifs or structural characteristics are present. Such ligands present complementary physicochemical interfaces to selectively interact with their cognate binding partner. The non-covalent complex which assembles can have a half-life of many hours [35] and it is the formation of this complex which may be directly or indirectly detected.

As with most discriminative bioanalytical techniques, clinical diagnostic applications have readily adopted antibody affinity based assays, most notably in the Enzyme Linked Immunosorbent Assay, or ELISA.
However, the time to complete these assays can be extensive due to the requirement to serially wash and incubate reagents in many stages. Relative to traditional methods (e.g. the mouse lethality assay) ELISA based technologies have been able to accelerate diagnostic output. However, the requirement of reagents and processing steps precludes the use of ELISA based methods for real-time analysis. Attempts to port assays from ELISA into a more portable format have been successful (e.g. chromatographic pregnancy test strips [36]). The processing simplicity is terrific; no washing is required and all the reagents are integrated into the chromatographic strip. But they compromise both sensitivity [37] and specificity [38, 39] relative to lab based ELISA methods.

Antibody proteins are not the only affinity ligands which may be employed, DNA sequences may also be targeted with high selectivity and sensitivity by a variety of means. Analogous to antibodies, DNA (when presented as a single strand) can coordinate/hybridise with its complimentary strand [40]. Saccharides, too, may be employed as affinity ligands [41], though such interactions can be comparatively low affinity with low specificity.

In principle, if a technology could be built around a high affinity ligand that can sense the formation of non-covalent specific physicochemical complexes, then the detection of biohazards may be instantaneous. Such affinity approaches are predicated on the fact that prospective environmental biohazards are known, and it has been possible to develop an affinity ligand to complex with it. The need for prior information is also common to bioinformatic approaches (e.g. DNA sequencing) as a sequenced entity must be known within a reference database in order to be attributed to any specific biological entity. So, for all approaches prior knowledge of biohazards is required.

For an environmental detection system, the overriding requirements of discriminative biological analysis within are; speed, specificity, sensitivity, stability and simplicity. Throughout the technologies illustrated above, there is a direct correlation between the specificity of derived analytical information and the time required to derive it. It is an enduring technical challenge to characterise biomaterials with satisfactory precision to enable real-time environmental biohazard monitoring.

1.1.3 Affinity Biosensors as Detection System Components

In 1962 Clark and Lyons reported the first biosensor [42]. They entrapped an enzyme, glucose oxidase, between semipermeable membranes over an oxygen electrode. The enzymatic oxidation of glucose yields hydrogen peroxide and gluconic acid, the former being selectively measured by the electrode (with the necessary assistance of the semipermeable membranes). This archetypal biosensor allowed electronic measurement of a biologically mediated process in order to detect and quantify the presence of a specific
analyte, glucose in this pioneering example. Since 1962, the advancement of broad scientific disciplines (genetics, physics, materials, etc.) has led to an elaborate divergence of scientific literature presenting a vast catalogue of putative biosensor components. As a technology domain biosensing has provided a conceptual workhorse for research and development bridging many scientific disciplines. In principle, all such biosensors may be simply defined as: a biologically mediated process, measured electronically to detect/quantify a material of interest. The principle components of a biosensor are illustrated schematically in Figure 1-1.

![Figure 1-1](image)

**Figure 1-1.** Schematic of principle biosensor components. Examples are included for indication and are not intended to be exhaustive. The sample matrix may be any sample type. The biological element need not necessarily be biologically synthesised and may be synthetically engineered as a bioinspired component (e.g. aptamers). The biological element is invariably immobilised upon a separate phase to the sample matrix. The biological element is appended to the transducer through which its behaviour may be electronically monitored via the means listed (though radiological transduction has also been investigated broad use of radioactive materials is not desirable due to the difficulties of waste management and notorious health implications). The requirement of signal processing depends upon the transduction mechanism employed but the visualisation/reporting of the analysis is universal.

Affinity biosensors are a class of biosensor technologies where the biological element employed has an inherent affinity for a specific material of interest, a cognate binding partner. Rather than detecting any intrinsic attribute of the analyte (i.e. inherent charge or chemical behaviour) the specific molecular complex formed (between affinity ligand and analyte) is the detected entity. The formation of a molecular
complex can be instantaneous and so, in the context of an environmental detection system, monitoring such interactions may allow real-time analysis, or near real-time as the sample must first be collected/partitioned in to an aqueous solvent. This sample may thus be continuously supplied to the sensor for analysis. Such automated and continuous collection of bioaerosols can thus be continuously analysed using an affinity biosensor.

Direct monitoring of affinity interactions, employing one affinity ligand for each analyte, allows affinity assays to become “reagentless”, i.e. at the point of analysis no additional reagents are required other than those integrated into the affinity sensing interface. Alternative strategies employing additional reagents (e.g. “sandwich” assays) have potential to increase sensitivity and specificity but this also increases system complexity (e.g. the requirement of fluidics to manipulate auxiliary reagents) and increases the time required to complete analysis and so precludes real-time monitoring. Reagentless analysis is desirable but assay requirements are more stringent, the affinity ligand must; be stably integrated into the technology interface, be specific for its analyte and demonstrate sufficient affinity for the analyte of interest. High affinity is not necessarily a prerequisite if the cognate analyte is anticipated to occur in high abundance, e.g. pollen allergens.

As the affinity complex is formed, in principle, a cognate analyte may be directly detected through numerous transduction modalities (i.e. any of those listed in Figure 1-1 and more besides, e.g. potentiometric, conductometric, field-effect etc.). Practically, however, mass sensors are the principle means by which affinity complexes are directly detected in real-time, on account of the variable physicochemical nature of analytes and confounding sample matrices (as they relate to biomaterials and environmental sample types, respectively). This makes direct electrochemical detection more challenging (i.e. variable and unpredictable) as the monitored properties may positively or negatively correlate with properties of the analyte (e.g. intrinsic charge) or environmental materials (e.g. salts) may change the behaviour of the interface itself (e.g. the electrical double layer). In contrast to electrochemical techniques, surface adsorbed mass can provide a uniform response which may be detected through either optical or mechanical means.

Optical surface plasmon resonance (SPR) biosensors [43] are a mature technology used extensively in research and development laboratories [44]. The SPR transduction principle is shown schematically in Figure 1-2. Integrated systems of SPR optics and the necessary microfluidics became commercially available in the mid 1980’s and have become indispensable research tools [45].
Figure 1-2. Schematic of the SPR transduction principle (Kretschmann configuration [46]). A wedge beam of monochromatic p-polarised incident light strikes a noble metal (i.e. gold) through a glass prism, where upon it is totally internally reflected. The reflected intensity of this light is not uniform and is quantified via CCD array. At a specific angle of incidence a minima is observed. Through momentum resonance photons of sufficient incident angle excite the electron density of the metal and the resulting oscillations (surface plasmons) propagate along the plane of the metal. The momentum required to excite the plasmon is directly proportionate to the refractive index of the material flanking the metal. Matter adsorbing within the evanescent field of the surface plasmon may moderate its resonance and thus materials may be detected by tracking the reflectance minima. The evanescent field decays exponentially from the plane of the surface, though it extends hundreds of nanometres into the probed volume. Aqueous biological buffers can be delivered to the sensing interface through fluidics and the immobilisation of affinity ligands upon the interface enables affinity based biosensing to be undertaken in real-time. SPR is typically sensitive to a refractive index change of \(1 \times 10^{-6}\) refractive index units [43]. This unit is employed to enumerate SPR response as “resonance units” which translates to a theoretical protein sensitivity of 0.91 ug.m\(^{-2}\) [43]. However, in the laboratory 5 ug.m\(^{-2}\) is practically more realistic accounting for sensor noise, i.e. variance due to the solvent and fluidic system, e.g. temperature, pressure, dissolved gases etc.).

Alternative optical and mechanical transduction modalities are amenable to real-time affinity sensing and have been commercialised. These are principally; optical waveguide lightmode spectroscopy (OWLS), biolayer interferometry (BLI), quartz crystal microbalance with dissipation (QCM-D) and surface acoustic wave (SAW). SPR theoretically provides greater sensitivity in direct biological interaction analysis with a sensitivity of 0.1 ug.m\(^{-2}\) compared to 1 ug.m\(^{-2}\) and 5 ug.m\(^{-2}\) for BLI and QCM-D respectively. SPR is also insensitive to variance in the conductance of sample matrices and analytes thought to affect SAW based sensors. However, while there are theoretical benefits to the use of SPR the commercial dominance of this biosensor modality is largely attributable to the development (and patent protection) of a key
component, a carboxymethyl-dextran based sensing interface [47]. This interface democratised the use of SPR technology allowing non-specialists to quantitatively study biological interactions in real-time. The commercial success of SPR evidences the critical component of any biosensor: the interface through which the sensor must operate.

1.1.4 The Role of the Affinity Biosensor Interface and its Component Parts

Much of the biosensor literature is scientifically elegant (e.g. single molecule detection) though direct implementation of such approaches in real-world applications is rarely evidenced⁴. It is surprising that, despite 56 years of intense biosensor research, the glucose oxidase sensor remains the principle real-world biosensor application (though it has evolved considerably in response to commercial drivers [48]). So, while the biosensor concept is readily assumed to deliver simple, sensitive and real-time analysis, the critical performance parameters of stability and specificity are frequently neglected. The impedance of technology translation is a consequence of performance variability outside of controlled laboratory environments.

The enduring technological development gap relates to the complexity of integrating biological elements as technological components. Biosensors must sustain the behaviour of these soft biological components which necessitates they be accommodated within a conducive thermodynamic environment. The materials which enable the various physical phenomena upon which biosensors depend are unnatural. Biological elements have no innate capacity to endure in such alien interfacial energetic environments. These soft and physically responsive biological elements can quickly change shape and thus the behaviour they are intended to impart may be compromised or entirely lost, and with it the benefit of the biosensor.

So, while principles of speed and simplicity may be engendered by ever divergent biosensor hardware, soft biological entities (as either components but also analytes) with their ill-defined interfacial physicochemical requirements, curtail system stability, specificity and sensitivity. For example, a hypothetical reagentless affinity assay applies a ligand to a sensing interface. The ligand is selected as it has an affinity for an analyte of interest which it binds with exquisite specificity as a free molecular species within a biological environment. However, once immobilised upon a chemically and physically alien interface such properties cannot be assumed to endure. After all, the binding interface of the ligand is inadvertently extended to include the entire interface to which it is bound. So even if the ligand does

⁴ Ostensibly because most biosensor literature seeks to evidence exquisite physicochemical principles rather than develop practical sensing devices per se.
maintain its binding behaviour, the interface upon which it sits must be equally sophisticated and resist any and all other interactions, biological or otherwise. The engineering principles to integrate technological components within biological systems by-design are still the subject of much research. The principle components of the affinity biosensor interface are displayed in Figure 1-3.

Figure 1-3. Schematic of an affinity biosensor interface. A) **Solvent**: without the solvent no selective biological interaction would be possible. B) **Analyte**: without a known analyte no affinity ligand may be developed. C) **Affinity ligand**: the properties of which underpin all key performance parameters, such as selectivity, sensitivity and stability. D) **Transducer**: the technology which interrogates the liquid phase invariably presents as a different phase itself, principally a solid. E) **Interfacial modifiers**: without modification water interfacial tension can be high (e.g. the surface tension of water at the gas-liquid interface is 72 mN.m⁻¹) which may easily subvert subtle biomolecular processes. Moderation of interfacial behaviours is commonly attempted through the immobilisation of polymers. F) **Assay interferent**: a material which has partial or complete physicochemical behaviour in common with the analyte. G) **Transduction interferent**: material and engages non-specifically with the interfacial system interfering with measurement. H) **Physical link**: connection between the affinity ligand and the transducer phase. Other solutes within the aqueous phase may also be of consequence (e.g. salts, surfactants, etc.). The arrow signifies the interrogation of the aqueous volume by undefined means, e.g. evanescent wave (e.g. SPR) or acoustic wave (e.g. QCW).
1.2 The Biological Recognition Element as a Component of the Affinity Biosensor Interface

1.2.1 Classes of Biological Recognition Element

Of all biopolymers used as recognition elements in affinity biosensor technologies proteins, and specifically antibodies, are the most widely reported [49]. Other biopolymers are reportedly able to support the requisite function (e.g. SOMAmers [50]) but the diversity of chemical functionalities supplied by amino acids, in combination with their three-dimensionally stabilised presentation upon structural scaffolds, provides functional advantages which have yet to be matched by synthetic alternatives, e.g. consistently high binding affinities for a broad range of analytes. This is principally a function of the interfacial surface area which proteins can support [51].

A schematic depiction of an antibody protein is presented in Figure 1-4 illustrating the most widely used format, the “IgG” or Immunoglobulin gamma. The IgG is composed of four polypeptide chains bound together through disulphide bonds. The composite Ig folds are arranged in pairs with the V_L-V_H dimer presenting the antibody binding site, or paratope. A single antibody molecule has two antigen binding sites. The rest of the structure is of consequence in the humoral immune system but for technological applications its presence may be useful as it increases the bulk of the overall entity which may facilitate crude manipulation strategies, e.g. immobilisation via physisorption.

![IgG Schematic](image)

**Figure 1-4.** IgG schematic showing individual Ig folds of the H chain (N-to C-terminus; V_H, C_H1, C_H2 and C_H3 in green) and the L chain (N- to C-terminus; V_L and C_L in red). The oligosacharides (in purple) orient between C_H2 domains of the Fc region.
All twelve domains of the IgG molecule are immunoglobulin (Ig) folds (Figure 1-5) and share sequence and structural homology. This domain unit of around 100 amino acid residues occurs extensively in nature serving many different functions across major phylogenetic branches [52-54]. The abundant utility of the Ig fold illustrates the versatility of this structural motif.

Figure 1-5. Schematic depiction of a human V\textsubscript{H} Ig fold domain derived using PDB file 1T2J and RasMol. Left: side view of the anti-parallel β sheet sandwich. Right: 90° rotation showing CDR peptide loops linking anti-parallel β strands at the top of the domain unit. CDR1 (pale blue), CDR2 (cyan) and CDR3 (dark green)

The physical dimensions of the core Ig fold are approximately 2.5 x 3.5 x 5 nm. IgG is a dynamic, mobile material (Figure 1-6) broadly of dimensions 15 x 7 x 3.5 nm [55]. It is not known how the dynamic structural motion supports in vivo function.
The conserved hydrophobic core of the Ig fold typically has a high intrinsic thermodynamic stability making it particularly suited to its role as a modular protein unit. This stability results from the anti-parallel β sandwich core structure and the disulphides which brace them [56]. These disulphides are not explicitly required for domain folding [57] but serve to condense the β sandwich [56] and so improve domain stability [58]. In some animals, such as camelids and cartilaginous fish, disulphide bracing is used more extensively in the VH domains resulting in further improvements in domain thermodynamic stability [59-61]. The stability of the Ig fold generally persists in the multimeric structures.

Protein engineers have sought alternative protein scaffolds to perform much the same function. Adaptive immunity has evolved other scaffolds [62]. The hagfish for example has the oldest adaptive immune system currently known which exploits “leucine rich repeat” motifs unrelated to the Ig fold. These can accommodate binding interface diversity and have potential for recombinant exploitation [63].

The adaptive immune system is not the only source of affinity materials. Protein scaffolds may be engineered synthetically and many researchers who led recombinant antibody technology in the 1990’s
developed this technology area to mitigate some of the issues of the early recombinant scaffolds. A popular feature of these alternative scaffolds is the absence of disulphide bonds (e.g. the α-helical “affibody” and ankyrin repeat protein [64]) though some scaffolds are homologous to the Ig fold (e.g. neocarzinostatin or “monobodies” based on the FnIII domain of fibronectin). For commercial reasons such investigations are typically geared to therapeutic markets [65] but their utility in biosensor technologies may be viable as these technologies aim to address many of the same practical/commercial challenges facing immunoglobulin scaffolds.

One possible limitation of proteins generally, however, is their dependence on aqueous environments to maintain their structural integrity and therefore binding specificity. Protein structure/stability relates directly to their thermodynamic environment. As protein recognition elements are frequently designed/evolved in aqueous environments they are predominantly limited to these conditions. However, as biological materials are derived by living things, and the solvent of biological systems is primarily aqueous, water is the most ideal solvent in which to interrogate specific binding properties of most biological materials. Other solvent matrices have been reportedly used as a medium for immunoassay (e.g. ionic liquids [66]) but water is the universal solvent for proteins and biological material in general and such deviations have yet to demonstrate functional benefits.

Synthetic recognition elements (composed of, e.g. nucleotides, saccharides and peptides) are an attractive option considering the relative complexities of biosynthetic approaches required of recombinant protein production. Oligonucleotide and peptide synthetic methods represent mature manufacturing technologies though their utility as specific biological recognition elements is largely unproven (though they can be generic ligands [67]). Of particular interest are aptamers which are nucleotide based and may autonomously fold into persistent structures. Aptamers may be used as straight forward binding elements [68] or may be engineered into self-reporting switches [69]. However, the core chemistry afforded by aptamers is constrained and in order to improve binding functionality in this class of binding element the incorporation of hydrophobic chemistries has been required [70]. The application of these elaborately engineered recognition elements into real-time applications is doubtful on account of the chain flexibility of the linear structure and the nonselective hydrophobic interactions which are likely to result. The platform technology in commercial development to exploit these reagents is explicitly avoiding this consequence by washing the bound species extensively (contributing to a 24 hour analytical processing time). This would render them unsuitable for real-time applications.

Molecularly imprinted polymers are another synthetic option. The selectivity of such materials appears inversely proportionate to the molecular mass of the analyte. They find utility in adsorbing low molecular mass species but as the scale increases the fidelity of interactions is lost. When at the length scale of
proteins, the overriding intermolecular forces may be anticipated to be non-specific. In principle, antibodies are molecularly imprinted polymers with the added advantage that, once the requisite polyamino acid sequence is defined, they self-assemble without need of template molecules.

Whole organisms have been reportedly used in the literature as affinity ligands (e.g. bacteriophage [71]) but the technological complexity of integrating discrete molecular scale recognition elements is challenge enough. It may be conceivable that the additional mass/bulk which may come with entities on the scale of phage may make deposition straight forward, but equally, maintaining the viability of such multicomponent molecular machines, within an energetically alien interfacial environment, seems ambitious.

1.2.2 Recombinant Antibody Engineering for Affinity Biosensor Applications

Antibodies are the archetypal affinity ligands. Sensitivity and specificity are the hallmark of these ligands and technologies have been built around them to exploit these properties. However, through recombinant engineering it may be possible to engineer these materials to provide more stable materials specifically engineered for controlled and reproducible integration into technologies. This may allow these proteins to be engineered to meet the need of future sensing technologies, rather than technologies being developed to compensate for the limitations of early antibody technologies (i.e. polyclonal and monoclonal antibodies). To this end, the intrinsic stability and controlled/reproducible manipulation of recombinant antibodies may be of benefit to broad technologies.

The scFv recombinant antibody format was first reported over thirty years ago [72, 73] and remains the most accessible format owing to the ready conversion of existing monoclonal hybridomas [74]. The scFv is a structurally minimised monovalent version of the conventional IgG format in which the variable (Fv) domains are genetically excised and fused into a single peptide chain. As a result of their accessibility much of the literature concerning recombinant technology is biased toward this scaffold.

Recombiant structures of the more recently discovered heavy chain antibodies, of cartilaginous fish (IgNARv) and camelids (V\text{H}H), can be further minimised to the V\text{H}i domain alone. The V\text{H}i and IgNARv are Ig folds but they have some identifying structural features/motifs. The camelid V\text{H}i is analogous to the V\text{H}i of the conventional IgG as it uses three CDR (complementarity determining region) loops to mediate binding. These domains are characterised by their extended CDR3 loops (relative to the V\text{H}i human and murine IgG). This characteristic loop serves two functions; the first obviously relates to antigen binding but the second is to help stabilise the domain. This is accomplished through the shielding of the putative hydrophobic V\text{L} interface. This interface also carries some characteristic mutations (known as the “V\text{H}i tetrad”) which
increase the hydrophilicity of the redundant V\textsubscript{L} interface. In contrast the IgNARv (thought to be an evolutionary relative of the IgG) has only two CDR loops which are analogous to the CDR1 and CDR3 of the camelid domain. The V\textsubscript{L} interface is entirely absent in the IgNARv domain and the CDR3 loop (though again of longer length relative to murine V\textsubscript{H} CDR3) is principally engaged in antigen binding.

Other single domain scaffolds have been derived from human Ig domains. A synthetic human V\textsubscript{H} domain has been developed as an alternative single domain scaffold with considerable inherent stability. While the V\textsubscript{H} domains of V\textsubscript{H}H and IgNARv antibodies provide a naturally robust domain scaffold the human V\textsubscript{H} scaffold has been purposefully reengineered. The representative V\textsubscript{H} “4D5” has particularly high inherent stability resulting from its Ig fold core. This specific example is so stable it can readily maintain its structure without any disulphide bonds. While the thermodynamic stability of these engineered V\textsubscript{H} domains is high its inherent propensity to aggregate in its denatured state is also high [75]. Through recombinant engineering this propensity has been reduced and the resulting scaffold demonstrates many of the properties of V\textsubscript{H}/IgNARv [76, 77].

Stability is clearly a property which can be engineered into recombinant antibody scaffolds, even scFv [78]. However, while the development of stable scaffolds increases the probability that the physical identity of the protein will survive technology integration [79] (i.e. they’re less likely to become physically distorted), the random orientation of their immobilisation can cause a problem if the loci of immobilisation occludes the binding site [80]. For predictable and reproducible immobilisation the chemistry of these recombinant proteins must be engineered [81].

Controlled, reproducible immobilisation should also benefit the miniaturisation of technology components, and transduction mechanisms. It is increasingly possible to monitor single molecule binding events. Where detection technologies have historically monitored net equilibria (e.g. ELISA) the ability to probe individual binding events seems ideal but stochastic transduction mechanisms require predictable binding behaviour. As a result the uniform immobilisation and bioactivity/purity of the protein become critical parameters within the assay. Through the manufacture of massively parallel transduction arrays it should be possible to overcome the issue of inherent bioactivity/purity through parallel redundancy. This may be pragmatic but binding interactions need to be tightly defined: analyte binding within stochastic arrays should principally exist it two states, i.e. bound and unbound. Any intermediate states may obfuscate the presence of the bound complex. This means that the immobilisation of the recognition elements must be as controlled as possible.
1.2.3 Limitations in the Manufacture of Engineered Recombinant Antibody Products

Antibody engineering technologies have matured and, while much proof of principle work has been undertaken to demonstrate the art of the possible with antibodies, modest wider adoption has resulted owing to the difficulty of producing useful quantities of functional recombinant proteins. For example, it became possible to excise extraneous domains from the conventional IgG format [82], but the \textit{in vivo} manufacture of such products hampered exploitation due to their low yields and poor stability. Such materials were academic workhorses to explore wider scientific principles. Early stability issues were principally related to the scaffold but even the inherently thermostable domain antibody format must be manufactured in a microbial host. While proteins may, in principle, be generally reengineered much needs to be done to transition these into real technological benefits as the manufacture of these engineered products is not yet routine.

Taking a step back, heterologous protein expression within microbial systems is, in general, quite an insult to the microbial host, which gets overrun with a tangle of molecular spaghetti with potentially catastrophic consequences. Such upregulated heterologous protein expression is a wholly unnatural process. The underlying problem is that, to some extent, all proteins have a propensity to aggregate. For a given condition each protein will have a maximal concentration at which it can persist as a stable discrete solvated entity. This concentration is a function of the dynamics inherent to the polypeptide, it’s solvent and solvated excipients establish an equilibrium where the peptide is statistically unlikely to encounter another molecular species and therefore avoid suffering irreversible, and energetically irresistible, interactions. Such aggregation may occur with otherwise stable proteins when their concentration is increased above such a threshold, and in artificial protein expression systems nascent species spontaneously aggregate as a statistical inevitability. Upregulated \textit{in vivo} expression constantly introduces new material to one side of the equilibria and if the concentrations of product, or any of the intermediates, are pushed out of equilibria then aggregation will occur manifesting as inclusion bodies. These are inherently toxic to the host. Protein expression is therefore quite an undertaking even when the product itself is soluble and stable.

The machinery of the host can be modified to yield more product. For example, it may be possible to co-express chaperonins or other catalytic machinery to boost the yield of the final product. However, the general applicability of such approaches is limited as native folding systems are already molecularly equipped to accomplish this task. The empirical, and largely arbitrary selection of, specific components to co-express comes at a cost to the overall expression system and to the yield of recombinant product as biomass is disposed to the coexpressed product. Switching to an alternative expression system can increase the degree of sophistication of the folding machinery and the manufacture of soluble, functional
material possible. This is a more pragmatic solution, but ideally the expression process should be kept as simple as possible [83].

Considering the above, the incorporation of explicitly reactive chemistry into in vivo expression systems can easily exacerbate an already toxic supramolecular jigsaw. The native palatte of amino acids contains a degree of redundancy in their chemical functionalities. Of the native amino acid functionalities cysteine has the most clearly differentiated chemistry. This may be targeted though electrophilic addition or substitution of maleimide or iodine functionalities respectively. Alternatively the reactivity of common functional groups may be modified as a direct result of their chemical environment. This phenomenon can underpin elaborate functions of enzymes [84] but it may be exploited by design through the relatively crude application of conjoined lysine residues [55]. The result is the concomitant reduction of amine pKa by co-locating lysine residues. This changes their chemistry and, therefore, their relative reactivity within the lysines of the mature protein. The otherwise simple repeat of lysine residues creates toxic products which impact host viability. Though the mechanism of toxicity remains uncharacterised cationic peptides are low yielding fusions [55] and such peptides are known to be antimicrobial so they may also be intrinsically toxic to their host. Both cysteine and lysine modifications, though derived from the native pallet of amino acids, are toxic to expression hosts. This is most likely a consequence of the very function for which they have been designed: chemical reactivity.

Fusion proteins have also been tested to increase the stability of expression. But, for biosensors, this has the consequence of adding extraneous protein mass (e.g. maltose binding protein) which cannot help its later application. Other Fusion partners are intended to add functionality (e.g. the SNAP tag). These are frequently too complex for microbial expression hosts to make in any great yield.

The overriding principle should be to keep it simple. Something new is needed to enable site-specific and reactive chemistry to be integrated within recombinant products without making the product too toxic to manufacture.

1.3 The Transducer as a Component of the Affinity Biosensor Interface

1.3.1 Transducers and their Constituent Materials

Biosensors exploit many different materials with a range of physicochemical properties, including; gold, silicone, quartz, diamond, graphene etc. These materials are selected as they mediate the niche physicochemical behaviour required in transduction. This is obviously a very necessary function, but their capacity to interface with biological materials is also an important parameter and, without exception, such
materials are alien and unnatural. The energetic response of the aqueous phase and its solutes is therefore unpredictable and frequently confounding.

Regardless of the atomic identity of the material involved the biological response to such materials is a consequence of one principle issue, a phase transition. Transducers are largely solid materials and biological materials exist within a liquid aqueous phase. This interphase system drives unpredictable and undesirable behaviour where selective bio-affinity interactions are required.

1.3.2 Strategies to Sustain the Fidelity of Biological Interactions at Affinity Biosensor Interfaces

When designing the affinity biosensor interface there are two key performance requirements; suppression of non-specific interaction to the interface, and preservation of the physicochemical identify of the affinity ligand upon which a reagentless affinity assay depends.

Binding sites must be accommodated within the sensing interface sympathetically. Antibody flexibility has been implicated in both binding function and stability [85, 86]. Compromising this dynamic flexibility can influence their ability to bind antigen [87, 88]. Protein paratope complementarity is also of great importance in binding interactions [89] and any remoulding of the variable domains will be detrimental to their binding activity [90]. The stability of immobilised binding sites can be affected as they may become distorted as a result of forces exerted by the underlying substrate [91]. A consensus for the successful accommodation of antibodies within biosensing interfaces is that these materials should be sustained in an environment comparable to that in which they have evolved to operate, i.e. water behaviour at the technology interface should mirror that in the bulk phase.

A universal assumption in the design of bio-fouling resistant interfaces is that functionalities incorporated to impart bio-resistance should be accommodated in sufficient density to present a contiguous physico-chemical barrier transecting the solid-liquid interface [92]. However, the requisite physico-chemical properties of this interface continue to be debated within the literature with proponents for both physical and chemical mechanisms of protein resistance ([93] and [94] respectively). The prolific and complex literature available concerning this subject belies the fact that water is a unique solvent with dynamic, multifactorial and competing molecular processes which propagate differentially across temporal and spatial length scales. The most obvious consequence is that observations within one interfacial system may well not be shared by any other. That said there is one universal observation which has generated consensus simply because the process can dominate biological systems and rapidly overwhelm them: hydrophobicity.
The concept of hydrophobicity is actually a misrepresentation. Classically, it describes the behaviour of non-polar functionalities (e.g. alkanes in an aqueous environment) and, contrary to literal interpretation, water is not scared of such non-polar solutes. There is simply a much greater energetic advantage for water to interact with itself. The solute is effectively excluded for not being water, not because it happens to have any property in itself. It is a largely non-specific process. This powerful force can easily overwhelm comparatively delicate biological processes and interfaces presenting hydrophobic functionalities are destined to be fouled non-specifically with co-solutes (like proteins, lipids, soot, soil etc.).

This phenomenon is driven by solvent entropy [95]. Any interface presenting hydrophobic functionalities reduces the degrees of freedom available to solvating water molecules in passive thermal processes (e.g. vibrational processes). This local retardation of water propagates away from the surface into the bulk solvent. So the hydrophobic functionality serves to coordinate a network of water molecules. The volume of water enduring such entropic injury is greater than a simple hydration layer. Not surprising then water will quickly depopulate the hydrophobic interface when practically any co-solute approaches the interface. This force can extend over tens of nanometres when two (large) hydrophobic surfaces approach in an aqueous solvent [96]. The concept of “interphase” water was invoked as a driver of non-specific biological adsorption by Vogler in 1998 [97].

In order to avoid being shunted into the hydrophobic interface the co-solute must interact strongly with water [92]. The generic term osmolyte has been applied to such materials and materials discovered in vivo which mitigate the degenerative impact of non-specific protein aggregation (i.e. heat stress or high osmotic pressure) are thought to operate through this mechanism (i.e. they coordinate water, maintain lubricity of cellular systems and in so doing prevent protein aggregation).

The modification of technological interfaces is not limited to the biosynthetic materials employed in vivo. A mature and empirically progressive technology area seeking to exploit hydrophilic behaviour is surfactant science. The palette of both hydrophilic functionalities developed for surfactant applications are numerous and provide a good tool kit to explore interface passivation. The catalogue of reported surfactant hydrophilic functionalities may be grouped into two categories; ionic and non-ionic. These describe the molecular mechanism through which the functional group interacts with water.

Ionic groups coordinate water molecules but they do so in a disordered manner and such water molecules are said to be “unfreezable”. This depends upon the electronic structure and bonding arrangement of the ionic species but biologically compatible ionic species tend to be large with low charge density (i.e. monovalent with large molecular/atomic radii). Non-ionic functional groups carry no formal charge but
their inherent polarity enables them to interact with water in a directional manner. Again this is a direct result of molecular structure but in contrast to the disorder found in ionic solvation water, non-ionic hydrogen bonds are directional and thus more constrained.

Ionic and non-ionic functionalities are thought to passivate interfaces to non-selective biological adsorption processes through two prospective mechanisms. They are both solvent mediated and therefore impart their effects indirectly. These are steric shielding (i.e. where functionalities bind water which then forms a steric barrier) and entropic shielding (i.e. where solvated functionalities are inherently disordered and their desolvation would reduce system entropy). The functionalities employed are simply an energetic scaffold onto which water may assemble, the key difference is in the role polymer disorder.

In pursuit of non-specific binding resistant surfaces the Whitesides group developed a self-assembling monolayer interface based on oligoethylene glycol [98]. The fundamental success of their approach related to their choice of polymer, polyethylene oxide (PEO also known as oligoethylene glycol or OEG, and polyethylene glycol or PEG depending on its molecular weight). PEO is considered a special or unique polymer and may be used in both aqueous and organic solvents. In aqueous environments PEG has some particularly advantageous properties owing to its elemental composition and bond arrangement. The polymer backbone consists of a simple repeating unit of saturated (carbon-carbon-oxygen)$_n$. The oxygen bond arrangement of this unit adopts a gauche conformation with bond angles similar to those in the surrounding water. This property, combined with the polarity of the oxygen, results in extensive interactions with the coordinated solvent. The lack of any explicit charge in PEG is a further boon considering one of the principle forces promoting non-specific surface fouling is electrostatic.

While the Whitesides interface provides continuous close coverage of the gold substrate to resist non-specific interactions [98], if a combination of different surface topologies are required for different analytes (e.g. proteins and cells [99] then this method is not readily suited to the production of zoned interface areas, i.e. a combination of 2D and 3D interfaces on the same bio-chip. Rubina et al. reported the zoned polymerisation of methacrylate (MA) promoted by ultra-violet irradiation [100]. In this approach the methacrylate monomer may carry any desired functionality (e.g. oligoethylene glycol [101] which may then be incorporated into the polymer. This functionality may include the antibody itself and has been shown to improve protein stability in the resulting 3D environments [102]. In such a process antibody immobilisation and polymer synthesis may be combined in light mediated arraying [102]. Such fabrication methods also enable both antibody functionalised hydrogel and planar interfaces to be deposited in discrete locations on the same arrayed biochip interface.
A resistant interface will not bind any material and this is the holy grail of materials engineering as this technical challenge is the prime limitation of bioengineering. This challenge is truly immense considering the inevitability of interfacial fouling when driven by solvent entropy. This is a result of the length scale over which technology interfaces must operate. This is an important consideration as water behaviour (specifically hydrogen bonding) can change significantly on a length scale of 1 to 10nm. This is exemplified by the hydration of hydrophobic solutes where a degree of solubility is possible at minor entropic cost as long as the solute has a radius <1nm. At this size the solute fits within the hydrogen bonding frameworks of the solvent. However, once the length scale increases hydrogen bonds must be broken to accommodate the solute and so solubility reduces considerably. A functional consequence for biosensor interfaces is that, on this length scale, they are vast. Even when hydrophilic functionalities are applied to the interface the impact on the first solvation layers is indistinguishable compared to hydrophobic hydration layers. So a surface, no matter how solvated it may be, presents an extensive and unnatural energetic boundary which may be expected to bind proteins. The only difference between hydrophobic and hydrophilic functionalities in this context is that hydrophilic surfaces bind with reduced affinity and may be practically removed through washing where hydrophobic adsorption is thought to be irreversible.

1.3.3 Limitations of Affinity Biosensor Interface Functionality Preventing Real-Time Detection

The propensity of current affinity biosensor interfaces to bind biological materials non-specifically is a long standing issue. The technical challenge is shared, in principle, with broader technology areas (e.g. marine fouling [103], medical implants [104] and window glazing [105]) but for affinity biosensing it is an acute blocker to real-world application. For example, in a marine context protein adsorption to ship hulls may be of little consequence if adsorbed species offer low mechanical resistance or, in a medical context if protein adsorption is transitory then dense aggregates are less likely to form. In affinity biosensing, however, resistance to binding must be complete, i.e. it must be undetectable. Any non-specific binding can give a false positive result and, while experimental controls can be incorporated to account for non-specific binding, the observed variance will increase experimental noise and false negatives will result as sensitivity is reduced.

Some literature reports are suggestive that zero adsorption has been observed [106], but the reported work tends not to monitor in situ interactions rather a measurement is taken once the surface has been washed, or a secondary probe is used to indicate adsorption of a specific species. This is inadvertently misleading. For affinity biosensors to function as a biohazard monitor, within a detection system as envisaged herein, in situ binding must be undetectable for all possible materials and at any concentration. This is a challenging technical goal.
1.4 Aims of this Work

This work concerns the engineering of affinity biosensor interfaces. The motivation is to develop new design rules to engineer this critical component of the affinity biosensor so they may be integrated within biological systems without perturbing native biological behaviour, principally non-specific adsorption/fouling, and optimise assay sensitivity and reproducibility. While components are necessarily developed separately the intention is to bring them together to understand how they perform in concert as a functional affinity biosensor interface. Throughout the work a Biacore 2000 surface plasmon resonance biosensor is used as a representative affinity biosensor. This specific SPR platform is used for reasons of opportunity though it is anticipated that any insights into interfacial design may find utility in affinity biosensor platforms more generally.

Results are presented in Chapters 3 to 5:

- **Chapter 3** addresses an interdisciplinary issue spanning protein manufacturing and protein technology integration. Protein engineering of recombinant antibodies is undertaken to introduce simple site-specific chemistry to facilitate protein integration within technologies, such as biosensors. Previously reported approaches have compromised the manufacturability of the product owing to the increased toxicity or insolubility of the engineered protein within the cellular host expressing it.

- **Chapter 4** takes a broad view across the published literature concerning biomaterial passivation to inhibit non-specific protein binding/fouling. Established reference interfaces (based upon zwitterionic or hydrogen bonding functionalities) are customised to incorporate molecular tethers (composed of polyethylene ether) for the immobilisation of biological recognition elements. Performance of the new surfaces is quantitatively evaluated in terms of fouling resistance, antibody immobilisation density and antigen binding performance.

- **Chapter 5** develops a novel affinity biosensor interface. Hydrophobic materials are used to coat the surface and self-organising oligoethylene glycol surfactants are used to passivate the interface to form a non-covalent surface coating. Molecular tethers (composed of polyethylene ether) are again integrated into the surface with a pendant ligand (i.e. biotin) as putative recognition element. The novel interface developed is a hybrid of covalent and non-covalent fabrication methods and is assessed for analyte sensitivity (i.e. streptavidin) and fouling resistance in undiluted animal serum.
Materials and Methods

2.1 Recombinant Antibody Engineering and Production

2.1.1 Bacterial Strains

*E. coli* strains XL1-Blue and BL21(DE3) were supplied by Invitrogen. XL1-Blue was used as a cloning strain to manipulate expression vectors such as pAK300 while BL21(DE3) was used for protein expression of the assembled vectors.

2.1.2 Plasmid Amplification and Purification

The expression vector pAk300 was gifted by the Pluckthun lab and is described by reference [107].

An individual *E. coli* XL1-Blue colony carrying a requisite plasmid (e.g. pAK300) was picked from appropriate selective media and inoculated into 5 ml of 2xYTGC (2xYT media containing 1 % glucose and 30 µg.ml\(^{-1}\) chloramphenicol). This culture was incubated overnight, shaking at 200 rpm at 37 °C. The overnight culture was centrifuged at 13000 x g for 10 minutes to pellet the cells and the supernatant discarded. Plasmid was isolated from cell pellets using a Qiagen mini plasmid preparation kit, according to the manufacturer’s instructions.

Agarose gels containing crystal violet were prepared by adding low melting point agarose (Invitrogen) at an appropriate concentration dependent on the size of DNA fragments that were to be resolved. The agarose was dissolved by heating in 150 ml of 1 x TAE buffer (40 mM Tris-acetate and 1 mM EDTA in H\(_2\)O) and then allowed to cool. Crystal violet solution was added to the molten agarose at a final concentration of 10 µg.ml\(^{-1}\). The molten gel was poured into a gel tray, allowed to set and placed into running buffer that consisted of 1 x TAE containing 10 µg.ml\(^{-1}\) crystal violet. Each sample was added to 5 x crystal violet loading buffer (100 µg.ml\(^{-1}\) of crystal violet and 30 % (w/v) sucrose in H\(_2\)O) and loaded into individual wells. Samples were electrophoresed at 75 V for 30 minutes or until the DNA products were suitably resolved.

Gel slices containing the appropriate products were excised from agarose gels. The DNA was then extracted from the agarose gel using the QIAquick® Gel Extraction Kit, in accordance with the manufacturer’s guidelines (Qiagen).
2.1.3 DNA Endonuclease Restriction

All restriction enzymes were obtained from New England Biosciences (NEB) and stored at -20 °C. Restriction enzyme digests were used to isolate DNA encoding genes of interest for sub-cloning purposes. All restriction enzyme digests were carried out on plasmid DNA or from DNA amplified using PCR according to the manufacturer’s instructions.

2.1.4 Pre-Cast Agarose Gel Electrophoresis

Pre-cast 1.2 % and 2.0 % agarose E gels (Invitrogen) were used to visualise purified plasmids from viable cells, restriction enzyme digests and DNA ligations. The gels were operated according to manufacturer’s instructions and DNA concentrations were estimated by running 5 µl of standard DNA marker (Invitrogen) at a known concentration.

2.1.5 Polymerase Chain Reaction Gene Amplification

Taq polymerase and associated reaction buffers were supplied by Invitrogen. The SNAP-tag™ commercially sourced from Covalys as coding DNA and used as a PCR template.

Standard PCR reaction conditions were used for the majority of PCR assays used in this work, with specific modifications if appropriate. In general a reaction mixture was prepared that contained 0.5 µl Platinum Taq DNA polymerase (Invitrogen), 0.5 µl of 100 µM forward primer, 0.5 µl of 100 µM reverse primer, 5 µl of 10 x PCR buffer (minus magnesium), 1 µl of 10 mM dNTP’s, 1.5 µl of 50 mM MgCl₂, and the reaction volume made up to 50 µl with sterile water. The DNA was then amplified using a PE 9600 Thermocycler.

2.1.6 Synthetic DNA Annealing

The DNA sequence encoding the chosen peptide sequences were synthesised as primers by Sigma Genesis. Oligonucleotides were dissolved in buffer 2 (NEB) to a working concentration of 650 µM. Complementary primers were combined, heated to 105 °C for 5 minutes and then incubated for 15 minutes at the melting temperatures and then snap cooled on ice to form a double stranded DNA stuffer fragment. These stuffer fragments were designed to provide overhanging DNA strands complementary to the restriction sites Nhe I and Pst I. The annealed stuffer fragments could therefore be ligated into pAK300 vector digested with Nhe I and Pst I and transformed into chemically competent E. coli cells. PCR was used to amplify the DNA sequence coding for the antibody and peptide. The PCR products were digested using
the restriction enzymes Nhe I and Pst I and visualised on a 2.0 % pre-cast agarose gel to ensure insertion of the correct DNA stuffer fragment.

2.1.7 DNA Ligation

scFv antibody sequences; OvA and OvE were generated by Isobel Atkin, M28 and B04 by Sarah Goodchild.

DNA products for ligation were quantified via electrophoresis on agarose gels with reference to known DNA standards. In general insert DNA was mixed with vector DNA at a standard ratio of 1:3 molar ratio of insert DNA to vector DNA with the exception of methods associated with construction of recombinant scFv libraries where a reaction mixture for each ligation typically contained 200 ng of cut vector to 20 ng of insert DNA. Each ligation reaction was then completed by the addition of 10 units of T4 DNA ligase (Roche), 1 x ligase buffer (Roche) and sterile water to a total volume of 20 µl. The reaction mixture was incubated for 16 hours at 16 °C in a Biometra Uno Thermoblock.

2.1.8 Transformation of E. coli

XL1-Blue or BL21 (DE3) E. coli competent cells (Stratagene) were used in accordance with the manufacturer’s recommendations (Stratagene). Transformed cells were re-suspended in 900 µl of room temperature regeneration media (2xYT media containing 10 mM (v/v) MgCl₂, 2.5 mM (v/v) KCl and 2 % (v/v) glucose). The transformed cells were incubated in a shaking incubator for 60 minutes at 37 °C. The transformed cells were finally plated out onto 2xYTGC plates containing appropriate antibody selection and incubated overnight at 37 °C.

Prior to transformation, ligated plasmid mixtures were de-salted by making up to 400 µl with distilled water, loaded onto a Microcon 30 spin column and centrifuged at 11000 x g for 8 minutes. The flow through buffer was discarded. This process was repeated, to give a total of three washes. After the final wash step, the centrifugation was repeated to ensure the final volume of DNA was reduced to 20 – 50 µl. The column was inverted in to a clean tube and centrifuged at 960 x g for 3 minutes to collect de-salted plasmid. XL1-Blue E. coli electrocompetent cells (Stratagene, Germany) were thawed on ice and combined with the de-salted plasmid in accordance with the manufacturer’s instructions. The cells and de-salted plasmid was transferred into an electroporation cuvette (2 mm gap, Bio-Rad Laboratories Ltd, UK) and pulsed in GenePulsar electroporator at 2500 V, 200 Ω and 25 uF. After electroporation the cells were re-suspended in 900 µl of room temperature regeneration media (2xYT media containing 10 mM (v/v) MgCl₂,
2.5 mM (v/v) KCl and 2 % (v/v) glucose). The cells were incubated at 37 °C, for 60 minutes, in a shaking incubator and then plated out onto appropriate selective media and incubated overnight at 37 °C.

2.1.9 Verification of Constructs by DNA Sequencing
Oligonucleotide sequencing services were supplied by MWG Biotech.

2.1.10 Isopropyl β-D-1-thiogalactopyranoside Induced Expression

_E. coli_ BL21 (DE3) clones containing each pAK300 expression vector were streaked out onto 2xYTGC plates and incubated overnight at 37 °C. A single colony was picked and used to inoculated 50 ml of 2xYTGC in a 250 ml culture flask and incubated overnight at 37 °C in a shaking incubator at 180 rpm. The culture was then centrifuged at 4000 x g for 15 minutes. The supernatant was discarded and the pellet re-suspended in 2xYT to a final OD<sub>600</sub> of 50.0. This inoculum was used to start fresh 50 ml shake flask cultures with an initial optical density of 0.1. The freshly inoculated _E. coli_ was cultured in 2xYT media to which had been added chloramphenicol to a concentration of 30 ug.ml<sup>−1</sup>, glucose to 0.1 % w/v and IPTG to 1 mM.

2.1.11 _E. coli_ Cell Harvest and Fractionation

Harvest of the expression cultures was undertaken at a defined time-point where expression products were only detectable by ELISA in the periplasmic fraction and not in the culture media. This time-point varied between recombinant antibodies (scFv-OVE and dAb-SA7). 25 ml of each 50 ml culture was extracted and cells separated by centrifugation at 13,000 x g for 10 minutes at 4 °C. The media supernatant was retained and the cells resuspended in 1.25 ml of ice-cold periplasmic extraction buffer (50 mM Tris-HCl pH 7.5 to which was added sucrose 20 % w/v and hen egg lysozyme 1 mg.ml<sup>−1</sup>). The re-suspended pellet was incubated on ice for one hour with periodic shaking. 1 ml of the suspension was transferred to microfuge tubes and centrifuged at 13,000 x g for 10 minutes at 4 °C. The supernatant containing the soluble periplasmic recombinant product was retained while the pellet, containing the _E. coli_ as spheroplasts, was resuspended in 1 ml of a cell lysis detergent formulation (BPER, Pierce). DNAse was added to degrade polynucleic acids which would otherwise turn the sample to a gel and prevent controlled manipulation via pipette. The lysates were incubated on ice for one hour in order for the DNAse to take effect before centrifugation at 13,000 x g for 10 minutes at 4 °C. The soluble fraction was extracted and the residual insoluble pellet solubilised in 8 M urea. Four fractions were, therefore,
produced for each culture; culture media, soluble periplasmic, soluble cytoplasmic, and insoluble cellular fractions.

2.1.12 Quantitative Comparison of Soluble Yields in Cell Extracts using ELISA

Immuno-2 HB 96-well micro-titre plates (Nunc) were coated with specific antigens. Typically soluble proteinaceous antigens were adsorbed at 10 μg.ml⁻¹ in PBS with 100 μl dispensed into each well. Following incubation at 5 °C overnight each plate was washed three times with PBS containing 0.05 % w/v p20 surfactant (PBST). 150 μl blocking buffer (2 % w/v skimmed milk powder (Marvel) dissolved in PBST) was added to each well with plates then incubated for at least 60 minutes at 37 °C. Each plate was washed three times with PBST and a solution containing antigen specific antibody was added as either a crude cell fraction (following method 2.1.11) or a dilution in blocking buffer. Bound antibody was typically detected using an appropriate anti-species secondary antibody conjugated to horseradish peroxidase (HRP). Antibodies used in this work included mouse anti-His HRP conjugate used at a 1:200 dilution in blocking buffer (100 μl/well). Development buffer, composed of 50 ml ABTS buffer, 2 ABTS tablets and 25 μl hydrogen peroxide was added (100 μl/well) and incubated for 30 minutes at 37 °C. Bound antibody was quantified by measuring the conversion of ABTS substrate to a coloured product based on readings at 414 nm in an automated plate reader (Anthos 2001). Where possible a dilution series of a purified product was used as a standard against which to quantify product yields in the cellular fractions. Where applied the standard was diluted in blocking buffer to a final concentration of 10 μg.ml⁻¹ and serially diluted down the plate. Appropriate negative controls were added to each plate to determine background readings.

2.1.13 Qualitative Comparison of Soluble Yields in Crude Cell Extracts using SPR

Following the Biacore 2000 user manual, ovalbumin protein was covalently immobilised within the carboxymethyl dextran hydrogel of the CMS (GE Healthcare) SPR sensor chip (via its amine functionalities using EDC/NHS chemistry). Ovalbumin was immobilised at a density of 1 ng.mm⁻² and the chip was sequentially exposed to the periplasmic extracts (from method 2.1.11). The binding rate of each fraction (specifically the relative gradient) was used to infer the concentration of scFv antibody within and between periplasmic fractions. Calculated values were corrected to account for the different mass of each protein product. The same CMS/ovalbumin chip was used for all periplasmic fractions being regenerated between exposures using a glycine buffer 50 mM pH 2.5.
2.1.14 Denaturing Polyacrylamide Gel Electrophoresis

Expressed soluble protein was evaluated for purity and confirmation of molecular weight by SDS-PAGE using 4-20 % Tris-glycine gels (Invitrogen), in accordance with the manufacturer’s guidelines. Samples were prepared by combining 20 μl of loading buffer with 20 μl of sample. The samples were incubated in a at 97 °C in a hot block for 4 minutes then pulsed in a microfuge for 5 seconds. Of each sample 30 μl was loaded and the gels electrophoresed in accordance with the manufacturer’s guidelines. The gels were removed from the cassettes and stained using ‘SimplyBlue’ SafeStain (Invitrogen) in accordance with the manufacturer’s guidelines or used for Western Blot analysis.

2.1.15 Western Blot

Samples separated by SDS-PAGE were transferred onto polyvinylidifluoride (PVDF) membrane (Invitrogen) in accordance with the manufacturer’s guidelines. Membranes were then incubated in blocking buffer (PBS containing 5 % w/v skimmed milk powder and 0.1 % v/v p20). Each membrane was then washed (3 x 1 min) in 0.1 % PBST (PBS containing 0.1 % v/v p20). The membranes were then probed using an antibody diluted in blocking buffer to a concentration of 10 μg.ml⁻¹ for 60 minutes at room temperature on a rocking platform. The blots were washed as described previously and incubated for 1 h at room temperature with shaking to selectively bind a horseradish peroxidase antibody conjugate (mouse anti-His HRP conjugate (Bio-Rad Laboratories)) which was diluted 1:2000 in blocking buffer. Blots were washed (3 x 1 min in 0.1 % PBST and 2 x 1 min in PBS). Protein bands recognised by the antibody were visualised by 3,3'-Diaminobenzidine (DAB) Peroxidase Substrate (SigmaFast™ DAB with metal enhancer) tablets according to the manufacturer’s instructions.

2.1.16 His Tagged IMAC Purification

The filter sterilised culture supernatant and periplasmic extract containing the expressed soluble scFv was buffer exchanged into PBS and concentrated down to ~30 mL using a VivaFlow 200 Flipflow Filtration. This concentrated sample was loaded onto a His-Trap column (GE Healthcare) using running buffer (40 mM w/v Tris-HCl and 750 mM w/v NaCl at pH 8.0), in accordance with the manufacturer’s guidelines. Nonspecifically bound protein was washed from the column using running buffer containing 50 mM imidazole. The soluble scFv was then eluted using running buffer containing 250 mM imidazole. Any remaining protein was then stripped from the column using running buffer containing 500 mM imidazole. Fractions collected from the column during the 250 mM imidazole elution step were combined and dialysed overnight against PBS to remove the imidazole. The dialysed proteins were then concentrated by
centrifugation using a concentration column (Vivaspin, 10 kDa MWCO: Sigma) in accordance with the manufacturer’s guidelines. The concentrated protein was quantified via BCA assay, aliquoted into volumes of 100 µl and stored at -80 °C.

2.1.17 Quantification of Protein Concentration

A standard dilution series of Bovine Serum Albumin (BSA) was produced by diluting an aliquot of BSA (2 mg.ml⁻¹, Pierce) in PBS to concentrations of between 1000 µg.ml⁻¹ and 10 µg.ml⁻¹. The protein standard (10 µl) was loaded in triplicate onto an Immulon-2 HB 96-well micro-titre plates (Nunc), into 140 µl of PBS. The sample to be quantified was diluted 1:10 and 1:50 in PBS and 10 µl was loaded in triplicate onto the ELISA plate into additional wells containing 140 µl of PBS. BCA reagents were prepared in accordance with the manufacturer’s guidelines and 150 µl added to each well of the ELISA plate containing either sample of standard BSA concentration. The micro-titre plate was incubated at 37 °C for 60 minutes. Absorbance readings were taken from the plate at 540 nm in an automated ELISA reader (Anthos 2001). The protein standards were used to prepare a concentration curve and used to calculate the concentration of the sample.

2.1.18 Reduction of the PDI Tag

Recombinant antibody in PBS at a concentration of 1mg.ml⁻¹ was reduced using 10mM TCEP for 10 mins on ice.

2.1.19 Thiol Selective Conjugation

PDI containing proteins were reduced and incubated on ice with a 10-fold molar excess of either maleimide or iodoacetic acid containing chemistries.

2.2 Covalent Biosensor Interface Production and Characterisation

2.2.1 Gold Surface Cleaning

For **ATRP polymer deposition**: Gold-coated glass SPR chips (Biacore SIA kit) were washed with ethanol before use. For re-use chips were cleaned using piranha solution (4:1 v/v conc. H₂SO₄ : 30 wt% H₂O₂. Caution! Piranha solution is a powerful oxidising agent that reacts violently with organic compounds; it
should be handled with extreme care) followed by a UV/O₃ clean (UVP PR-100 UV-Ozone photoreactor, 1000 seconds).

2.2.2 Self-Assembled Monolayer Deposition

For ATRP polymer deposition: Chips were then initiator coated by soaking in a 1mM solution of disulphide ATRP initiator (Prochimia) in ethanol for 18 hours. Samples were washed with ethanol, water and dried under a nitrogen stream.

2.2.3 Polymer Deposition by Aton Transfer Radical Polymerisation

Materials: Poly(ethylene glycol) methacrylate (PEGMA-OH-360, Mn 360, contains 500-800 ppm MEHQ inhibitor), poly(ethylene glycol) methyl ether methacrylate (PEGMA-OMe-300, Mn 300, contains 100 ppm MEHQ and 300 ppm BHT inhibitors), 3-sulphopropyl methacrylate potassium salt (KSPMA, 98%), [2-(methacryloyloxy)ethyl] trimethylammonium chloride solution (METAC, 80 wt. % in H₂O, contains 600 ppm MEHQ inhibitor), 2,2'-dipyridyl (bpy, ≥99%), (+)-Sodium L-ascorbate, (≥98%) and copper(II) bromide (99%) were purchased from Sigma-Aldrich (Gillingham, UK). Ethanol (Absolute, 99.99%), hydrogen peroxide (100 vol, >30% w/v) and ammonia solution (35% wt. %) were purchased from Fisher Scientific (Loughborough, UK). 2-methacryloyloxyethyl phosphorylcholine (MPC) was purchased from Vertellus Specialities (Middlesborough, UK). Water was deionised using an Elga Option 4 system.

Formulation of MeOEGMA mixture for polymerisation by ARGET ATRP: PEGMA-OMe-300 was first purified by passing through a short column of basic alumina to remove inhibitors. PEGMA-OH-360 was found to degrade on a basic alumina column and so was not purified before use. A solution of PEGMA-OMe-300 (9.0g, 30mmol, 0.9 eq) and PEGMA-OH-360 (1.2g, 3.3mmol, 0.1 eq) in water (10ml) was deoxygenated by bubbling through nitrogen for 15 minutes. To the solution was added CuBr₂ (4.0 mg, 18μmol, 5.4x10⁻⁴ eq), 2,2'-dipyridyl (bpy, 7.0 mg, 45 μmol, 1.4x10⁻³ eq) and sodium ascorbate (300 mg, 1.5 mmol, 3.6x10⁻² eq) and the mixture was stirred under nitrogen to dissolve the solids, forming a brown solution. The mixture was then transferred to a vial with a screw-top lid.

Formulation of PMPC-POEGMA mixture for polymerisation by ARGET ATRP: A solution of MPC (0.404 g, 15 mmol 0.9 eq) and PEGMA-OH-360 (0.595g, 1.7 mmol, 0.1 eq) in water (2.5 ml) and methanol (2.5 ml) was deoxygenated by bubbling through nitrogen for 15 minutes. To the solution was added CuBr₂ (4.0 mg, 18μmol, 1.1x10⁻³ eq), 2,2'-dipyridyl (bpy, 7.0 mg, 45 μmol, 2.7x10⁻³ eq) and sodium ascorbate (300mg,
1.5mmol, 9.0x10^{-2} eq) and the mixture was stirred under nitrogen to dissolve the solids, forming a brown solution. The mixture was then transferred to a vial with a screw-top lid. MPC and PEGMA-OH-360 were used as received.

**Formulation of PMPC mixture for polymerisation by ARGET ATRP:** These polymerisations were conducted as for PMPC-PEGMA above, except that the mixture of MPC and PEGMA-OH-360 was replaced by MPC (5.0g, 17mmol, 1 eq).

**Formulation of charge-balanced mixture for polymerisation by ARGET ATRP:** A solution of METAC (4.94g of a 78.8 %wt solution in water, equivalent to 3.89 g, 19 mmol, 0.45 eq dry METAC), KSPMA (4.61 g, 19 mmol, 0.45 eq) and PEGMA-OH-360 (1.50 g, 4.2 mmol, 0.1 eq) in water (8.95 ml) was deoxygenated by bubbling through nitrogen for 15 minutes. To the solution was added CuBr_{2} (4.0 mg, 18 μmol, 4.3x10^{-4} eq), 2,2’-dipyridyl (bpy, 7.0 mg, 45 μmol, 1.1x10-3 eq) and sodium ascorbate (300 mg, 1.5 mmol, 3.6x10^{-2} eq) and the mixture was then transferred to a vial with a screw-top lid. METAC and KSPMA were used as received. METAC solution concentration was determined by extended drying at 110 C.

**General surface-initiated polymerisation procedure:** Initiator coated SPR chips were polymer coated by immersion into the polymerisation solution under air in a screw-top vial (although the screw-top remained closed when samples were not being added or removed). After the desired polymerisation time (typically 2-3 minutes for all systems), SPR chips were removed, washed well with water (and methanol, if used as a polymerisation solvent) and dried under a nitrogen stream.

### 2.2.4 Physical Characterisation (Goniometry, Ellipsometry, XPS and AFM)

**For ATRP polymer characterisation:** Ellipsometric measurements were conducted using a rotating analyser ellipsometer (Gaertner Scientific, USA) with a 632.8 nm laser at an angle of incidence of 70°. Ellipsometric parameters for the gold surfaces were fitted by measuring the samples after coating with initiator, and assuming an initiator thickness of 1.6 nm. All organic layers were assumed to have a refractive index of 1.50.

XPS spectra were obtained using a VG Scientific Escalab 5 Mark II X-ray photoelectron spectrophotometer, with an unmonochromated Al source. A Shirley background was used, and peaks were fitted using XPSPEAK 4.1.

AFM scratch profile analysis was undertaken by Dr S Connell (Leeds University). 300 nN force (0.1 V setpoint) was used to sweep deposited polymer and SAM from the gold surface. A force of 3000 nN was required to sweep the gold from the underlying glass support (Figure 2-1) and so 300 nN used to
characterise polymer thickness was not sufficient to disrupt the gold layer (<0.1 nm penetration anticipated).

![AFM line section of SIA gold surface scratch profile showing the depth of the gold coating upon the glass substrate being 46 nm.](image)

**Figure 2-1.** AFM line section of SIA gold surface scratch profile showing the depth of the gold coating upon the glass substrate being 46 nm.

### 2.2.5 Non-Selective Adsorption Screening

Chips were warmed up to room temperature (30 minutes on the bench in a sealed pouch or glassware) and docked in the BIAcore 2000 instrument after being mounted if necessary. A prime function was run in HBS-EP buffer to prepare the chip and fluidics of the instrument for optimal performance. A sensorgram was initiated at a flow rate of 5 µl min⁻¹ in detection mode for all 4 flow cells and ran for 1 hour or until the change in response units over 10 minutes was less than 10 RUs. The chip was then normalised using the normalise function with BIAcore normalisation solution. The chip was subsequently primed again and flushed to remove any residual normalisation solution from the surface and fluidics.

Three methods were written using BIAcore control software to ensure each repeat in the series for a surface chemistry was comparable to the same chip number of another surface chemistry. Every command in each of the three methods was the same for all cycles only the location and therefore the material that was injected was changed.
The order of NSB materials for each method is shown below in Table 2-1.

<table>
<thead>
<tr>
<th>Method 1 (chip 1)</th>
<th>Cycle 1</th>
<th>Cycle 2</th>
<th>Cycle 3</th>
<th>Cycle 4</th>
<th>Cycle 5</th>
<th>Cycle 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc1</td>
<td>HEWL</td>
<td>Shp</td>
<td>Fib</td>
<td>IgG</td>
<td>-</td>
<td>BSA</td>
</tr>
<tr>
<td>Fc2</td>
<td>Fib</td>
<td>HEWL</td>
<td>-</td>
<td>Shp</td>
<td>BSA</td>
<td>IgG</td>
</tr>
<tr>
<td>Fc3</td>
<td>Shp</td>
<td>IgG</td>
<td>HEWL</td>
<td>BSA</td>
<td>Fib</td>
<td>-</td>
</tr>
<tr>
<td>Fc4</td>
<td>-</td>
<td>Fib</td>
<td>BSA</td>
<td>HEWL</td>
<td>IgG</td>
<td>Shp</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Method 2 (chip 2)</th>
<th>Fc1</th>
<th>IgG</th>
<th>BSA</th>
<th>Shp</th>
<th>-</th>
<th>HEWL</th>
<th>Fib</th>
</tr>
</thead>
<tbody>
<tr>
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<td>BSA</td>
<td>-</td>
<td>IgG</td>
<td>Fib</td>
<td>Shp</td>
<td>HEWL</td>
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<td>Shp</td>
<td>-</td>
<td>IgG</td>
<td>BSA</td>
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<tr>
<td>Fc4</td>
<td>Shp</td>
<td>HEWL</td>
<td>IgG</td>
<td>Fib</td>
<td>BSA</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Method 3 (chip 3)</th>
<th>Fc1</th>
<th>Fib</th>
<th>-</th>
<th>HEWL</th>
<th>BSA</th>
<th>Shp</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Fc2</td>
<td>IgG</td>
<td>Shp</td>
<td>BSA</td>
<td>HEWL</td>
<td>-</td>
<td>Fib</td>
<td></td>
</tr>
<tr>
<td>Fc3</td>
<td>-</td>
<td>BSA</td>
<td>Fib</td>
<td>IgG</td>
<td>HEWL</td>
<td>Shp</td>
<td></td>
</tr>
<tr>
<td>Fc4</td>
<td>BSA</td>
<td>IgG</td>
<td>-</td>
<td>Shp</td>
<td>Fib</td>
<td>HEWL</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-1. Details of the order of NSB materials in the three methods used to assess each surface chemistry.

For each surface chemistry three chips were assessed. This ensured that each constituent of the NSB panel was a first exposure twice across the three chips. The NSB panel (hen egg white lysozyme (HEWL), bovine serum albumin (BSA), fibrinogen, immunoglobulin G (IgG) and sheep serum) was made up in HBS-EP (Hepes buffered saline with EDTA and surfactant p20 0.005 wt%), this was also the running buffer to ensure refractive index changes due to a change of buffer were minimised. Each chip underwent the same preconditioning.

The assay was carried out under continuous flow conditions at a flow rate of 5 µl.min⁻¹ for both injections of NSB material and dissociation. Each assessment had six cycles so each flow cell was exposed to all of the NSB materials in a differing order. Each flow cell was dealt with individually within the cycle and all four flow cells were regenerated together.

2.2.6 Protein Concentration Quantification via Refractive Index Measurement

The refractive index of a protein solution will register as a stepped change in SPR sensors as they only monitor refractive index and the area interrogated by the SPR sensor is three-dimensional. This means
that any proteins within this thin but three-dimensional aqueous area will be detected by the SPR sensor. This bulk refractive index change may be used to confirm the sensitivity of SPR sensing interfaces; the bulk step change should be of similar magnitude across all surfaces if they are mutually sensitive. However, should a chip coating be of significantly greater thickness the bulk response will be reduced as the sensitivity of the evanescent SPR field decays exponentially with distance from the gold layer. The relationship between protein concentration and refractive index change was verified. The linear relationship of a porous carboxymethyl hydrogel, sourced commercially as a CMS sensor chip (GE Healthcare), is presented in Figure 2-2. The sensitivity of each chip may therefore different, dictated by the thickness of the coating upon the gold, but the proportionality of response should be consistent within the same chip and those of similar sensitivity.

Figure 2-2. Bulk refractive index change, of a CMS chip baring a carboxymethyl dextran hydrogel, in response to BSA at different concentrations.

2.2.7 Antibody Immobilisation on Carboxyl Containing Surfaces

A 35 µl aliquot of EDC:NHS was injected across flow cell 1 at a flow rate of 5 µl min\(^{-1}\) to activate the surface, this was followed by an injection of 15 µl of ethanolamine to deactivate the surface. Flow cell 1 acted as the blank. EDC:NHS was prepared from stock solutions made up according to BIAcore protocols.
and mixed 1:1 v/v using the control software immediately prior to injection to minimise degradation and error between operators.

A 35 µl aliquot of EDC:NHS was injected across flow cells 2, 3 and 4 at a flow rate of 5 µl min\(^{-1}\) to activate the surface. This was followed by a Rb anti-OVA diluted to 100 µg ml\(^{-1}\) in acetate 4.5 and 70 µl injected across flow cells 2, 3, and 4. This was followed by 15 µl of ethanolamine to deactivate the surface.

### 2.2.8 Antibody Physisorption on SIA or Hydrophobic Surfaces

Rb anti-ovalbumin was diluted to 100 µg.ml\(^{-1}\) in acetate 4.5 and 70 µl injected across flow cells 2, 3, and 4 at a flow rate of 5 µl.min\(^{-1}\).

### 2.2.9 Antibody Immobilisation on Hydroxyl Containing Surfaces

Hydroxyl containing surfaces were derivatised by submersion in a solution of 100mM DSC in DMF for 16 hours. The chip was subsequently washed in anhydrous DMF and sonicated for three minutes prior to submersion into a solution of 100mM biotin-PEG-amine in DMF. The biotinylated chip was then rinsed and sonicated again with a final wash in ethanol before being dried under vacuum and stored under an argon atmosphere.

Once the chip was docked in the SPR system and equilibrated, streptavidin was diluted to 100 µg.ml\(^{-1}\) in HBS-EP and 225 µl injected across flow cell 1 at a flow rate of 5 µl.min\(^{-1}\), this created an interface free of antibody and served as a negative control.

Biotinylated rabbit anti-ovalbumin was diluted to 100 µg.ml\(^{-1}\) in HBS-EP with the addition of 100 µg.ml\(^{-1}\) streptavidin and 225 µl injected across flow cells 2, 3, and 4 at a flow rate of 5 µl.min\(^{-1}\). HBS-EP was used as the antibody diluent instead of acetate 4.5 as the low pH may adversely have affected the biotin streptavidin affinity reaction.

### 2.2.10 Antibody Activity Screening

Ovalbumin was diluted to 1 mg.ml\(^{-1}\) in HBS-EP and aliquoted before freezing. For each chip a new aliquot of ovalbumin was thawed and further diluted to 100 ng.ml\(^{-1}\), 1 µg.ml\(^{-1}\), 10 µg ml\(^{-1}\) and 100 µg.ml\(^{-1}\) in HBS-EP. The ovalbumin dilutions were then transferred to sealed glass vials to minimise evaporation.

Three chips were used to assess each surface type. The experimental design is shown in Table 2-2.
<table>
<thead>
<tr>
<th>Chip 1</th>
<th>Cycle 1</th>
<th>Cycle 2</th>
<th>Cycle 3</th>
<th>Cycle 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc 1</td>
<td>100 ng.ml⁻¹</td>
<td>1 µg.ml⁻¹</td>
<td>10 µg.ml⁻¹</td>
<td>100 µg.ml⁻¹</td>
</tr>
<tr>
<td>Fc 2</td>
<td>100 ng.ml⁻¹</td>
<td>1 µg.ml⁻¹</td>
<td>10 µg.ml⁻¹</td>
<td>100 µg.ml⁻¹</td>
</tr>
<tr>
<td>Fc 3</td>
<td>1 µg.ml⁻¹</td>
<td>10 µg.ml⁻¹</td>
<td>100 µg.ml⁻¹</td>
<td>100 µg.ml⁻¹</td>
</tr>
<tr>
<td>Fc 4</td>
<td></td>
<td>10 µg.ml⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chip 2</th>
<th>Cycle 1</th>
<th>Cycle 2</th>
<th>Cycle 3</th>
<th>Cycle 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc 1</td>
<td>1 µg.ml⁻¹</td>
<td>10 µg.ml⁻¹</td>
<td>100 ng.ml⁻¹</td>
<td>100 µg.ml⁻¹</td>
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<th>Cycle 1</th>
<th>Cycle 2</th>
<th>Cycle 3</th>
<th>Cycle 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc 1</td>
<td>10 µg.ml⁻¹</td>
<td>100 ng.ml⁻¹</td>
<td>1 µg.ml⁻¹</td>
<td>100 µg.ml⁻¹</td>
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<td>100 ng.ml⁻¹</td>
<td>1 µg.ml⁻¹</td>
<td>100 µg.ml⁻¹</td>
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<tr>
<td>Fc 3</td>
<td>100 ng.ml⁻¹</td>
<td>1 µg.ml⁻¹</td>
<td>100 µg.ml⁻¹</td>
<td>100 µg.ml⁻¹</td>
</tr>
<tr>
<td>Fc 4</td>
<td></td>
<td>1 µg.ml⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-2. Order of ovalbumin concentrations for each chip of all surface chemistries.

For each chip there was a predefined order that the antigen concentrations passed over the flow cells. This was to ensure that for each chip the lower three concentrations passed across a different flow cell first. The order was changed for each chip to ensure results were not biased by using the same flow cell and therefore flow dynamics for any one concentration. This reduced the possible error from instrument limitations.
2.2.11 Whole Serum Binding Titration Assay

Using a Biacore 2000 a sensor chip was docked and equilibrated in an appropriate buffer (e.g. HBS-N composed of 10 mM HEPES pH 7.4, 150 mM sodium chloride). The chip was then exposed sequentially to a series of three injections of running buffer to equilibrate the injection loop and reduce artefacts introduced by the auto-sampler. The flow rate used was 20 ul.min\(^{-1}\) and injections were limited to 150 seconds. A dilution series of whole serum was made up from a frozen aliquot for each separate experiment. Whole serum being diluted three-fold into running buffer until the proportion of serum present represented only 0.0002 % v/v of the sample. Sequential injection was undertaken following a buffer blank and then the thirteen injections of increasing serum concentration, terminating after exposure to whole serum.

2.3 Non-Covalent Interface Production and Characterisation

2.3.1 Aqueous Deposition of Self-Assembled Monolayers

SIA gold surfaces were used as received (GE Healthcare) and simply placed within a 1 ml aqueous solution to which had been added; 10mM C12E6, 10mM L-77 and 1 μl of an alkanethiol (either undecanethiol or \(1H,1H,2H,2H\)-Perfluorodecanethiol). The surfactant/oil mixture was emulsified and applied to the SIA chip without pre-treatment. The submerged chip was then incubated at 25 °C for at least 16 hours in a shaking incubator. Chips were then sonicated for 150 seconds, rinsed in ethanol and air dried immediately prior to use.

2.3.2 PEG Lipoamide-Biotin Tether Immobilisation on Gold

The above method (2.3.1) was modified. The tether was deposited at a defined concentration in the aqueous surfactant solvent without the alkanethiol oil. This was incubated for a period of one hour before alkanethiol oil was emulsified within the same surfactant formulation and incubated for a further 16 hours as per method 2.3.1.

2.3.3 Serum Binding Assay

Prepared surfaces were exposed to whole rabbit serum to which had been added appropriate surfactants to a final v/v of 0.05 %. Injections were at a flow rate of 20 ul.min\(^{-1}\) and of 600 second duration.
2.3.4 Streptavidin Binding Assay

Prepared surfaces were exposed to a solution of streptavidin 10 ug.ml$^{-1}$ to which had been added appropriate surfactants to a final v/v of 0.05 %. Injections were at a flow rate of 20 ul.min$^{-1}$ and of 600 second duration. Binding selectivity was tested by injection of BSA under identical conditions and concentrations.
Assessing In Vivo Toxicity of Recombinant Antibodies Engineered to Contain Site-Specific Chemistry for Orientated Immobilisation within Affinity Biosensor Interfaces

3.1 Aim of this Chapter

The work herein aims to develop a recombinant antibody peptide fusion tag to facilitate orientated immobilisation of the antibody within real-time affinity biosensor technologies. The chemistry incorporated within the peptide tag should be native to the expression host (i.e. composed of natural amino acids), principally to reduce complexity/cost. It should also yield a soluble product with low inherent toxicity within the expression host or functional yields may be compromised.

Six C-terminal immobilisation tags are compared for scalable manufacture in the common recombinant expression host Escherichia coli. A novel tag, denoted the “Protein Disulphide Isomerase” (PDI) tag, contains two cysteine residues which may be bound together in vivo in a dithiol bond. Should this yield a soluble product the intrinsic thiol chemistry will provide a selective yet simple functionality to target for subsequent conjugation/immobilisation. This novel peptide fusion tag is compared with a series of known fusion tags with previously reported limitations (e.g. inherent toxicity and/or low solubility).

3.2 Design and Construction of Recombinant Antibody Expression Vectors

3.2.1 Selection of Peptides as Prospective C-Terminal Fusions

C-terminal fusions have been selected in preference to N-terminal fusions due to the reported observation that binding site occlusion may result from fusion at the N-terminus [108]. Five peptide tags were selected as prospective scFv fusions to provide site-specific functionality. These are listed in Table 3-1.

The Hexa-Histidine tag (HIS) was used as a positive control. The inherent toxicity of this peptide fusion is assumed to be low and should be well tolerated [109]. Affinity ligands for HIS are also readily available which may be used to detect expression in crude cell fractions (e.g. monoclonal antibody “HIS-1” and nickel chelating agents). The HIS tag is also included in all other C-terminal fusions as a potential affinity purification tag [109].

A single cysteine residue (CYS) can be incorporated into the peptide but unpaired thiols are known to be toxic within E. coli [110]. It was anticipated that this fusion peptide would be toxic to the host and was therefore used as a toxic reference system.
<table>
<thead>
<tr>
<th>C-terminal tag</th>
<th>Added Molecular Mass (Da)</th>
<th>Amino Acid Sequence (single letter code)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexa-Histidine (HIS)</td>
<td>840</td>
<td>HHHHHH</td>
<td>Bell [109]</td>
</tr>
<tr>
<td>Single Cysteine (CYS)</td>
<td>944</td>
<td>HHHHHHCE</td>
<td>Torrance [111]</td>
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<tr>
<td>Biotin Mimetic Peptide (BMP)</td>
<td>2540</td>
<td>ASPCHPQFPRCYALQH</td>
<td>Das [112]</td>
</tr>
<tr>
<td>Biotin Acceptor Peptide (BAP)</td>
<td>3165</td>
<td>ASGGGLNDIŒAQKIEWHELQHHHHH</td>
<td>Schatz [113]</td>
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<tr>
<td>Thiol oxidoreductase Substrate (PDI)</td>
<td>2376</td>
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<td>Ruddock [114]</td>
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<tr>
<td>SNAP tag™ (SNAP)</td>
<td>21511</td>
<td>ASMDKDCEMKRTTLDSLPGKLELSGCEQGLHEI...</td>
<td>Kufer [115]</td>
</tr>
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</table>

Table 3-1. Fusion peptides selected for comparison as C-terminal tags to facilitate site specific modification of recombinant antibodies. Reactive residues from each peptide tag have been underlined. Where possible, results data throughout this chapter have been colour coded.

The Biotin Mimetic Peptide (BMP) has been reported to have affinity for streptavidin [112] and the mature periplasmic product should also contain a disulphide bond within the 11 residue peptide. Previous applications of this tag have resulted in low soluble yield. The resulting insoluble inclusion bodies can be readily purified and refolded in vitro to generate a functional product [112]. This tag is a reference system, again demonstrating product insolubility.

The Biotin Acceptor Peptide (BAP) is a synthetic substrate of native *E. coli* biotin ligase (BirA). This peptide substrate can be biotinylated in vivo when BirA is coexpressed [113]. The efficiency of biotinylation of expressed product is not complete as the nascent peptide must be biotinylated within the cytoplasm before it is secreted into the periplasmic space. However, a significant proportion of the product may be biotinylated in vivo as a post translational modification using this method should a suitable expression host be used [116].
The protein disulphide isomerase (PDI) tag has not been applied as a fusion tag before. It is known to be an effective substrate for disulphide bond isomerases (both eukaryotic Protein Disulphide Isomerase and prokaryotic DsbC) [114] and has been used to study enzyme kinetics in vitro. When fused to a periplasmic protein the mature expression product should contain a disulphide bond if it is indeed an efficient substrate of bacterial DsbC. This has not been reported as a recombinant fusion but it is intended to function as a comparison peptide for the BMP fusion and may provide soluble product with a selectively activatable disulphide bond.

The final tag is an enzyme fusion which has been commercialised under the name SNAP tag™ (SNAP). The SNAP tag is an engineered enzyme based on O⁶-alkylguanine-DNA alkyltransferase [117]. As a native enzyme it enables DNA repair by sacrificing itself to remove DNA adducts [118]. The native mechanism is mediated by a reactive cysteine [119]. While unpaired cysteine residues are known to be toxic, when sequestered within a protein domain this inherent toxicity may be reduced. The SNAP tag is a modified form of this enzyme exploiting this sacrificial mechanism to accept derivatives of benzylguanine as synthetic substrates. Through this enzymatic route anything conjoined to the N7 position of the benzylguanine can be site specifically conjugated to the protein. The SNAP tag is a mammalian protein and is relatively large in comparison to the expressed scFv.

### 3.2.2 Design and Construction of Modular E. coli Expression Vectors

In order to compare the C-terminal fusions effectively it was necessary to develop a modular expression system. To this end a bacterial lac operon based expression vector known as pAK300 [107] was modified to enable the construction of a homologous series of expression vectors containing the same scFv sequence fused to different C-terminal tags. A schematic of the amended vector is shown in Figure 3-1. The initial pAK300 vector contains a scFv composed of connected V₅ and V₆ domains flanked with N-terminal FLAG tag (inserted as an antibody affinity tag [120]) and C-terminal hexa-histidine tag (nickel affinity tag for purification [109]). The parent vector was modified to include additional unique restriction sites (specifically NheI and PstI endonuclease sites) to enable the modular insertion of fusion peptides. Each additional restriction site elongates the translated peptide by two amino acids but the impact of these peptide extensions was mitigated through the selection of restriction sequences which provide the most optimal expression codons in E. coli and are, therefore, unlikely to limit the efficiency of translation of mRNA transcripts (codons selected according to [121]).
The coding DNA for C-terminal fusions was either assembled from chemically synthesised DNA (Method 2.1.5) or via PCR amplification from a reference sequence with customised primers (Method 2.1.5). Regardless of the source, the product was ligated directly into SfiI/HindIII cut pAK300 (Methods 2.1.2, 2.1.3 and 2.1.6). Using these assembly methods the PDI, BAP, BMP and SNAP tags were constructed. For the CYS tag a single cysteine residue was included as the terminal residue following the HIS tag (this vector was constructed by I. Atkin).

Figure 3-1. Color-coded schematic of the customised pAK300 cloning vector and recombinant periplasmic scFv product. The full sequence of the original pAK300 vector backbone is provided in ref. [107]. The customisation to this vector is detailed in Appendix A.
3.3 Comparison of *In Vivo* Toxicities and Soluble Yields of Peptide Fusion Constructs when Expressed in *E. coli*

3.3.1 Characterisation of Unmodified scFv Expression in *E. coli* Shake Flask Culture

The recombinant antibody chosen to compare the expression of C-terminal fusion tags was a scFv format. This was selected for its known compatibility with *E. coli* as an expression host and the product is soluble and tolerated by the host (I. Atkins, unpublished data). This scFv, known as OVE, binds a nontoxic model protein analyte: chicken egg ovalbumin. To characterise expression behaviour of the unmodified HIS tagged scFv an *E. coli* expression system (strain BL21 DE3) was used in small scale 50ml shake flask cultures (Method 2.1.9).

An induced culture (containing IPTG and 0.1% glucose) was compared with control cultures (containing 0.1% and 2.0% glucose) and growth rates were monitored to determine the relative impact of scFv expression on the viability of the growing culture. Though the scFv was originally selected as a well-tolerated recombinant protein in *E. coli*, the growth profiles (Figure 3-2) show a significant difference in the rate of growth in induced and suppressed cultures ($t=480$ unpaired t-test $p=0.002$). IPTG induced expression significantly reduces bacterial growth rate and this starts to take effect 300 minutes after inoculation ($t=360$ unpaired t-test $p=0.018$). This demonstrates that expression of this scFv has an inhibitory effect on cell growth. The 0.1% glucose culture (without IPTG) follows an identical growth profile as the culture containing 2% glucose ($t=480$ unpaired t-test $p=0.319$).

The nascent scFv is directed to the bacterial periplasm by the pelB leader sequence [122]. This is necessary owing to the structurally essential disulphides which require post translational formation/shuffling to ensure the final product is folded correctly [122]. As the scFv product accumulates in the periplasm it can leak out of this compartment due to as yet uncharacterised and likely complex mechanisms [123]. Whatever the mechanism, the tagged products passively leak through the outer membrane and into the growth media and this may preclude accurate comparison of *in vivo* tag solubility/yield. It is also possible that the different C-terminal fusions will stress or permeate the outer membrane of the expression host in different ways and to different extents. Such interference may be minimised by harvesting the expression products before the point at which the periplasm loses integrity. This should ensure that any difference in product yield is attributable to *in vivo* tag solubility/toxicity within the periplasm of the expression host and not its ability to permeate the outer membrane.
Figure 3-2. Effect of IPTG induced scFv expression on the growth rate of *E. coli* strain BL21 DE3. Three cultures carrying the HIS tagged pAK300 scFv expression vector were cultured in a base media of 2xYT broth supplemented with; 2.0% glucose (---), 0.1% glucose (-----), and 0.1% glucose plus 1mM IPTG (-----). Error bars represent one standard deviation from the mean where n=3 for OD600 from the same culture.

**Figure 3-3** profiles the increasing concentration of the scFv product in both the periplasm and the culture media with time (detected and quantified by HIS tag ELISA: **Method 2.1.11**). At 150 minutes after inoculation the product is detectable within the periplasm. At 330 minutes the product is detected in the culture media, having leaked from the periplasm.
Figure 3-3. Quantification of scFv accumulation over time within two *E. coli* culture extracts; media (——) and cellular periplasm (——). The HIS tagged products were quantified through ELISA by comparison against a purified control of known concentration. The scFv product accumulates within the periplasm and leaks into the culture media. The selected harvest point for comparison of C-terminal fusion peptide solubility/toxicity is indicated by a dashed line (——) when the product is present within the periplasm but has not yet permeated the outer membrane of the expression host. Error bars represent one standard deviation from the mean where n=3 within the same ELISA.

To determine comparative soluble yield across the C-terminal tags the time point to harvest the periplasmic fraction was identified as being 240 minutes following inoculation. It is anticipated that this will reduce experimental complication resulting from the differential impact of each tag on the expression hosts outer membrane.

In order to generate a baseline expression yield, against which to compare the C-terminal tags, identical cultures to those presented in Figure 3-2 were harvested at t=240 and fractionated into four extracts; culture media, cellular periplasm, cellular cytoplasm and insoluble matter. The concentration of HIS tagged scFv product in each fraction was quantified using a histidine tag mediated ELISA with reference to a known standard (purified scFv of known concentration). Figure 3-4 shows the yields of scFv in the four culture extracts.
Figure 3-4. Yields of scFv with HIS tag in four culture extracts; media, periplasm, cytoplasm ("BPER"), insoluble matter ("urea"). Three culture conditions are shown each with a base media of 2xYT broth supplemented with; 2.0% glucose (■), 0.1% glucose (□), and 0.1% glucose plus 1mM IPTG (▲). Error bars represent one standard deviation from the mean where n=3 from the same ELISA. Only the IPTG induced culture contains substantive scFv product.

Media fractions of all three cultures contained no detectable product. This confirms that when cultures are harvested at t=240 the expression product does not significantly compromise the outer membrane of the expression host and periplasmic products are retained.

Between the culture conditions tested there are differences in periplasmic extracts: the induced culture has a high level of product compared to both controls. The control cultures containing glucose at 0.1% and 2.0% yielded detectable expression products in similar quantity (unpaired t-test p=0.37). As the difference between induced and suppressed cultures is significant (unpaired t-test p=<0.0005) it is evident that catabolite repression has either relaxed or is not sufficient to prevent IPTG induction. This means the comparison of in vivo tag toxicity in this expression system should still be viable as products will still accumulate under IPTG induction.

Interestingly the cytoplasmic BPER extract also contains a relatively high level of scFv. This may be the result of incomplete periplasmic extraction if some cells are not converted to spheroplasts. Some carryover between periplasmic and cytoplasmic extracts is to be expected as spheroplasts may carry some product with them by simple trapping within the cell pellet when centrifuged. It is also possible that the scFv product is folding within the cytoplasm before it is secreted into the periplasm. The level of carryover
or cytoplasmic folding has not been determined and therefore there may be limited significance in any difference, however, should the cytoplasmic extract contain more product than the periplasmic extract then it may be reasonable to assume that the product was not translocated across the cytoplasmic membrane efficiently and instead accumulated within the cytoplasm. This was not the case for the HIS tagged product in these control cultures and most of the detectable product occurs within the periplasm.

It is also interesting that the urea extract of the induced culture also generates a detectable response in ELISA. This suggests that the scFv peptide may fold into a functional product when diluted into the ELISA blocking buffer. This is unlikely considering the complexity of in vitro refolding of immunoglobulin products [112] and it is more likely that the solubilised inclusion bodies start to precipitate or bind non-selectively to the polystyrene ELISA plate when the concentration of urea is reduced through the threefold dilution of the 8M urea extraction buffer (when added to the assay buffer and further diluted down the ELISA plate). It may be possible that refolding does occur but it is not possible to discriminate between selective and non-selective binding within this data.

Following the quantitative characterisation of HIS tagged scFv expression products within the four culture extracts, the relative impact of different C-terminal tags on host viability and product soluble yield may be determined.

### 3.3.2 Comparison of in vivo Toxicities of the expressed scFv C-Terminal Fusions

When cultures harbouring the C-terminal fusion vectors (from section 3.2) are grown under identical conditions (as characterised in 3.3.1) the relative toxicities of the expressed peptides toward *E. coli* may be determined. Figures 3-5 and 3-6 show the growth profiles of *E. coli* cultures carrying the vectors for the C-terminal fusion peptides. In Figure 3-5 the cultures contain 2% glucose and no IPTG. They exhibit similar growth profiles showing no significant differences between cultures (t=480 one-way ANOVA p=0.931). As the lac operon does not provide absolute control over the expression of the scFv product (as shown in 3.3.1) leaky expression may be expected to occur. Therefore, at these low concentrations, none of the tags exhibit acute toxicities in excess of the leaky lac expressed HIS tagged product. The observed reduction in growth rate is likely a result of media exhaustion (Sezonov et al. reported a related observation and detected the depletion of amino acids from LB media [124]). This may also be explained by the aggregation of scFv expression even if it is at low abundance. Whatever the reason it is a universal phenomenon across all suppressed cultures so comparative testing through IPTG induction is a viable approach.
Following the observation that IPTG induced scFv expression reduces culture growth rate (Figure 3-3) it is not surprising that all IPTG induced cultures exhibit reduced growth rates as a result of induction (Figure 3-6 relative to Figure 3-5).

Comparing the induced cultures in Figure 3-6 there are significant differences between them (when t=480 one way ANOVA p<0.0005). Relative to HIS tagged scFv the CYS tagged product was the most toxic of the five fusions and cellular growth rate was significantly affected after (t=480 unpaired t-test p<0.0005). BAP and SNAP tags significantly reduced the peak cell densities achieved relative to the HIS product (t=480 minutes unpaired t-tests p<0.0005). This suggests that the accumulation of these products reduces culture viability to a greater extent than the HIS tag alone. Considering peak cell densities of both BMP and PDI tags were not significantly different to HIS (at t=480 and t=360 unpaired t-tests p=0.102 and p=0.064 respectively relative to HIS t=480) it is possible that the amount of expressed scFv may be comparable to the original scFv fusion when harvested at t=240 when the cultures have identical OD₆₀₀nm.

Peak cell densities occur when the culture enters stationary or death phase. The CYS expression culture does not appear to enter the stationary phase as may be expected when culture nutrients are exhausted and instead immediately enters the death phase. The only cultures which do not decline significantly within this 480 minute incubation are HIS and BMP cultures. Paired t-tests between OD₆₀₀nm values at t=420 and t=480 are as follows; HIS p=0.107, PDI p=0.019, BAP p=0.003, BMP p=0.289, CYS p=0.003 and SNAP p=0.010.
Figure 3-5. Growth rates of *E. coli* BL21 DE3 cultures carrying scFv expression vectors with different C-terminal fusion peptides with expression suppressed through the presence of 2.0% glucose; HIS (---), PDI (---), BAP (---), BMP (---), CYS (---), SNAP (---). Error bars represent one standard deviation from the mean where n=3 from the same expression culture.

Figure 3-6. Growth rates of *E. coli* BL21 DE3 cultures with IPTG induced expression of scFv with different C-terminal fusion peptides with expression induced through the presence of 1 mM IPTG; HIS (---), PDI (---), BAP (---), BMP (---), CYS (---), SNAP (---). For convenient comparison with Figure 3-6 the y axis is scaled identically. Error bars represent one standard deviation from the mean where n=3 from the same expression culture.
3.3.3 Comparison of Soluble Yields of scFv C-Terminal Fusions

To compare the soluble yield of each fusion tag expression cultures were harvested 240 minutes after inoculation and fractionated into four extracts; media, periplasm, cytoplasm and insoluble extracts. The concentration of each tagged scFv product was again quantified using a histidine tag mediated ELISA with reference to a known standard (purified scFv of known concentration). Figure 3-7 plots the yields of the fusion tags in each of the four extracts. In addition to the harvest at 240 minutes another was undertaken at 24 hours to determine the longer term effect of product expression. These cultures were composed of the residual culture volume (25ml) from the 240 minute harvest.

The only fusion tag not to generate any detectable soluble product was the SNAP tag. This indicates that either the histidine tag was not accessible to the immunoconjugate used in the ELISA, or that there was no soluble and functional scFv product present. All other tags generated a detectable soluble yield which may be quantitatively compared though this assumes the accessibility of the histidine tag is uniform across the different products.

At t=240 the culture media fractions contained no detectable expression products. At this time point the yields across the induced periplasmic extracts follow the order HIS>PDI>BAP>CYS=BMP and the differences are significant (unpaired t-tests; HIS>PDI p=0.001, PDI>BAP p=0.011, BAP>CYS=BMP p=0.003 and CYS=BMP p=0.121 respectively). While HIS, PDI and BAP appear most abundantly as soluble products in the periplasm, CYS and BMP appear most abundant within the cytoplasm. Unpaired t-tests between periplasmic and cytoplasmic extracts are as follows; HIS p=0.001, PDI p=0.005, BAP p=0.036, CYS p=0.024 and BMP p=0.118. Most of the products are more abundant in the periplasmic extract but the CYS is the only one to be significantly more abundant in the cytoplasm. The difference in yield is significant and suggests that this product is not efficiently secreted by the Sec machinery or that it is insoluble in the periplasm. This appears to be the case with BMP also but the difference is insignificant.

Following 24 hours incubation the cultures will be nutritionally exhausted. In addition, most of the cultures had entered death phase after 480 minutes and so cells will have been yielding to the various stresses induced by their expression products. Under these conditions, factors which reduce cellular viability are counter-selected. This may explain the apparent loss of all soluble products other than HIS, PDI and BAP (Figure 3-7 B) and the fact that these products occur in greatest concentration in the culture media and not within the remaining cultured cells.
Figure 3-7. Yields of scFv with different C-terminal tags harvested in four culture extracts; culture media, cellular periplasm, cytoplasmic BPER, and insoluble urea extracts. HIS tagged reference data from Figure 3.5 is included for comparison. Harvests from twelve cultures are shown, each culture having a base media of 2xYT broth and are presented as pairs of induced (0.1% glucose + 1mM IPTG) and suppressed (2.0% glucose) cultures; HIS induced (■) and suppressed (□), PDI induced (■) and suppressed (□), BAP induced (■) and suppressed (□), BMP induced (■) and suppressed (□), CYS induced (■) and suppressed (□), SNAP induced (■) and suppressed (□). Error bars represent one standard deviation from the mean where n=3 from the same ELISA plate. A) Yields 240 minutes after inoculation. B) Yields 24 hours after inoculation. The y axis scale is identical for convenient comparison of A) and B).
Denaturing polyacrylamide gel electrophoresis was used to compare periplasmic and insoluble urea fractions harvested from the induced and suppressed expression cultures to determine whether the products of low soluble yield were present as insoluble aggregates within the expression host. The same periplasmic fractions from Figure 3-7 were applied within Figure 3-8. There are many contaminating products in these crude preparations. However, there are visible bands which are attributable to the anticipated scFv products in IPTG induced cultures. As anticipated from the data in Table 3-1 the masses of the scFv products are different due to the different fusion peptide masses. Identical volumes of extract were applied to each lane of the gel which allows qualitative comparison. The suppressed culture extracts are qualitatively the same while there are differences between the induced culture extracts. The CYS expressing culture has greater general coomassie staining intensity suggesting the cells within this culture were not in the same physiological state as the other cultures. This may relate to the fact the cells were suffering from the innate toxicity of the CYS tag. By eye the HIS product appears to be the most abundant while PDI, BMP and BAP products are qualitatively equivalent. Neither CYS nor SNAP tagged products are visible though they may be present within the background.

The denaturing PAGE gel for insoluble culture extracts (8M urea) is shown in Figure 3-9. The control cultures are qualitatively identical. Products attributable to the anticipated expression products are observable in all IPTG induced cultures. This demonstrates that, to some degree, all expressed products are insoluble when using this expression method. Again, the intensity of the bands qualitatively indicates the abundance of the insoluble products. Qualitatively, band intensity follows the order CYS>Snap>BMP>BAP>PDI>HIS.

It is possible that the ELISA based quantification of the soluble extracts may have been affected by differential accessibility of the histidine tag. According to Figure 3-7 the BAP tag should be 10 fold less concentrated and it is clear from the relative intensities in Figure 3-8 that this is unlikely to be the case or it would most likely not be observable in this gel (as is the case with the CYS product). It is possible that the amount of soluble protein present and the functional binding activity of the products may not correlate (i.e. there may be a soluble product but it may not retain its binding function). This would result in a low functional activity (as determined by ELISA) and high protein abundance (as determined by SDS-PAGE). To explain this discrepancy a label free surface plasmon resonance assay was used for comparative analysis of periplasmic expression products (Figure 3-10). As this method is mass sensitive (inferred from changes in refractive index) the binding responses have been normalised to account for the difference in mass of each fusion tag (according to Table 3-1). The results are quite different to the ELISA result and broadly follow the same trend present in SDS-PAGE (Figure 3-8).
Figure 3.8. Denaturing PAGE of the periplasmic extracts harvested from the cultures illustrated in Figures 3-6 and 3-7. Lane 1: BenchMark™ His-tagged standard, IPTG induced cultures as follows; Lane 2: HIS, Lane 3: PDI, Lane 4: BAP, Lane 5: BMP, Lane 6: CYS, Lane 7: SNAP. Suppressed cultures containing 2.0% glucose as follows; Lane 8: HIS, Lane 9: PDI, Lane 10: BAP, Lane 11: BMP, Lane 12: CYS, Lane 13: SNAP. Colour coded arrows indicate the presence of bands attributable to identifiable scFv products.

Figure 3.9. Denaturing PAGE of the insoluble urea extracts harvested from the cultures illustrated in Figures 3-6 and 3-7. Lane 1: BenchMark™ His-tagged standard, IPTG induced cultures as follows; Lane 2: HIS, Lane 3: PDI, Lane 4: BAP, Lane 5: BMP, Lane 6: CYS, Lane 7: SNAP. Suppressed cultures containing 2.0% glucose as follows; Lane 8: HIS, Lane 9: PDI, Lane 10: BAP, Lane 11: BMP, Lane 12: CYS, Lane 13: SNAP. Colour coded arrows indicate the presence of bands attributable to identifiable scFv products.
Figure 3-10. Soluble periplasmic concentration of each scFv fusion, relative to the HIS tagged standard, determined through a label-free surface plasmon resonance assay upon a Biacore CMS SPR chip. **A)** Schematic of SPR data generated to illustrate the gradient of refractive index change used to compare soluble yields relative to the HIS tagged control. A schematic is provided in place of the original data as the sensor system used to generate the data was no longer available (it became obsolescent and was disposed of). **B)** Soluble concentration of each fusion is expressed as a percentage of the HIS tagged scFv control. The protein concentration within the periplasmic extract is inferred through comparison of binding rates (i.e. comparison of the gradient of refractive index change upon a CMS SPR sensor surface carrying the antigen, hen ovalbumin). Error bars represent one standard deviation from the mean of the calculated binding gradient of each extract (n=3 and the periplasmic extracts were the same as those compared in Figures 3-7 and 3-8).

**Note:** Calculated values for each extract have been corrected to account for the relative masses of the C-terminal tags as without such correction concentrations of larger protein tags (such as the SNAP tag) would be disproportionately higher owing to their greater mass.
The order of soluble yield based on the SPR assay is \textbf{HIS=PDI>BAP>BMP>CYS>SNAP} (unpaired t-tests; HIS=PDI \(p=0.082\), PDI>BAP \(p<0.0005\), BAP>BMP \(p=0.008\), BMP>CYS \(p=0.010\), CYS>SNAP \(p=0.015\)). A soluble and functional SNAP tagged product is detectable in the periplasmic fraction which is the most notable difference between the ELISA assay and the SPR assay. However, while the trend in soluble yield is similar between ELISA and SPR assays, the proportional differences in yields are quite different. The SPR data is label free and therefore more reliable and it shows that the PDI tag is the highest yielding soluble fusion being insignificantly different from the HIS tag product. The next most soluble tag is the BAP tag though there is a significant reduction in soluble yield.

\subsection*{3.3.4 Application of Low Toxicity, High Yielding Fusion Peptides to Other Recombinant Antibodies}

A selection of other scFv sequences were used to test the general applicability of the PDI and BAP tags. For convenience, only the soluble periplasmic yields were compared in ELISA and relative yields are shown in Table 3-2. Across the scFv tested, the relative yield of these two tags mirrors the trend observed in 3.3.3: the PDI tag generates a more abundant soluble product relative to the BAP tag.

Considering the disparity observed between the relative abundance of PDI and BAP products determined by ELISA and SPR (Figures 3-7 and 3-10 respectively) the difference in yields is unlikely to be as drastic as appears in Table 3-2.
Table 3-2. Qualitative comparison of soluble PDI and BAP fusion tag yields when applied to alternative scFv sequences listed in Appendix B. Tabulated values relate to the dilution factor at which expression products remain detectable (i.e. yield a signal in excess of the background plus three standard deviations of the assay background) using a histidine tag ELISA to probe the periplasmic extract harvested from a 24 hour expression culture. OVE scFv is included for comparison as it is the reference scFv used in expression screening presented in Figures 3-2 to 3-10.

Following the successful application of the PDI tag to other scFv it was also applied to a single domain antibody (dAb) and compared to a HIS tag equivalent. The growth profiles of these expression cultures are shown in Figure 3-11: relative to HIS the PDI fusion does not reduce culture growth rate.
Cultures identical to those profiled in Figure 3-11 were harvested (at t=360 before expression impacted culture viability) and fractionated into four culture extracts. Figure 3-12 indicates the relative yields of the two products across these extracts. The main contrast with the data presented for the comparative scFv data (Figure 3-7) is that the main soluble product appears in the cytoplasmic extract and not in the periplasmic extract. Interestingly the yield of the PDI tag in the cytoplasmic extract is reduced relative to the HIS. This was observed in the scFv products (Table 3-2) also and partially explained by the accessibility of the tag used in the ELISA to compare product yields. This is likely to be the case here also but the relative impact would have to be verified through a label free assay.
Figure 3-12. Qualitative comparison of dAb yields with HIS or PDI C-terminal fusion fractionated into four culture extracts; culture media, cellular periplasm, cytoplasmic BPER, and insoluble urea. Harvests from four cultures are shown, each with a base media of 2xYT broth and are presented as induced (0.1% glucose + 1mM IPTG) or suppressed (2.0% glucose) pairs; HIS induced (■) and suppressed (□), PDI induced (▲) and suppressed (▲). The titrating dilution corresponds with the highest dilution of the culture extract which still generated an ELISA response in excess of three standard deviations above the background of the assay. A) Relative yields 360 minutes after inoculation. B) Relative yields 24 hours after inoculation. The y axis scale is identical for convenient comparison of A) and B.)
Interestingly the **HIS** product within the cytoplasmic extract is abundant. This suggests that the product is not toxic to the cells, which in turn suggests the product does not aggregate within the host. However, it also suggests that the product is not efficiently secreted by the Sec-pathway which suggests the dAb product folds within the cytoplasm [125]. The benefit of having a soluble cytoplasmic product is that it may be produced in large quantity. However, when the **PDI** tag is applied within this context, it reduces the amount of soluble product. This would suggest that the C-terminal tag itself is not as well tolerated as the **HIS** tag within the cytoplasm of *E. coli*. The causes for this may be numerous, such as the upregulation of proteases which degrade the product. The benefits therefore of the **PDI** tag relate to periplasmic expression only, certainly in this strain of expression host.

In order to confirm that the increased soluble yield observed within the cytoplasmic extracts was a result of low aggregation of the product, denaturing polyacrylamide gel electrophoresis was undertaken of all extracts (**Figure 3-12**). The most notable result is that, in contrast to the scFv, dAb expression does not lead to significant aggregation (**Figure 3-13 D**). Across these gels no products are visually detectable, which was unexpected considering the abundance of soluble material detectable in ELISA (**Figure 3-12**).

This dAb was selected for subsequent work due to the evident stability of this class of scaffold. To validate the *in vitro* application of the **PDI** tag dAb expression was scaled up. In large scale 250 ml shake flask culture an IMAC purified yield of 23 mg was achieved.
3.4 Site Specific and Covalent \textit{in vitro} Conjugation of a Recombinant Antibody via the Disulphide Containing C-Terminal Peptide Fusion “PDI”

3.4.1 Optimisation of Selective Disulphide Reduction

The \textit{in vitro} conjugation of the thiol functionalities within the PDI tag requires the reduction of its disulphide bond. There are four disulphide bonds within the selected PDI tagged dAb. The disulphide within the C-terminal tag is likely to be the most solvent accessible and so selective activation may be possible through a relatively simple process: controlling the temperature of the reduction reaction. Avoidance of modifying the three other disulphides is essential because they are structural: one maintains the immunoglobulin fold motif, and two stabilise the Complementarity Determining Regions of the
domain. Unintentional reduction of these may be indicated by a loss of binding function or loss of solubility of the protein.

Figure 3-14 illustrates the impact of incubation with the reductant TCEP (tris(2-carboxyethyl)phosphine). It was hoped that by incubating the reaction at 0°C that the immunoglobulin fold would remain compact and so prevent the TCEP from accessing internal disulphides. However, it is apparent that binding function drops with longer incubation with TCEP. After 320 minutes the activity of the antibody appears reduced. In order to preserve the activity of the antibody the reaction must be stopped once the PDI loop has been reduced.

**Figure 3-14.** Reduction of 5A7-PDI antibody binding activity with increasing incubation time with reductant TCEP (10mM) at 0°C. Relative binding activity (i.e. TCEP treated dAb/untreated dAb) derived by His tagged ELISA of soluble 5A7-PDI antibody binding to immobilised protein hen egg lysozyme. This data provides indication only and the significance of any differences between time intervals and residual activity are not known due to a practical issue affecting ELISA data which precludes quantitative discrimination (incomplete plate washing/conjugate contamination).

In order to compare the efficiency of each of the TCEP incubations in Figure 3-14 it was possible to use the same monoclonal antibody used in the quantitative ELISA’s in section 3.3.3 (HIS-1). In the ELISA assay performed previously the relative response generated by this histidine affinity ligand was related to the accessibility of the tag. It was subsequently possible to use this observation to infer the extent of PDI
reduction by comparing the relative accessibility of the histidine tag. The assumption being that if the PDI tag were reduced then the relative intensity of an affinity based western blot would increase.

A western blot of the products from a time series of TCEP incubations was prepared following requisite non-reducing denaturing polyacrylamide gel electrophoresis (a replica gel can be seen in Figure 3-15). The denaturing PAGE shows that the bands are qualitatively similar and so it can be assumed that the lanes are all loaded with comparable quantities of protein. In contrast the HIS-1 alkaline phosphatase immunoconjugate used to develop the western (Figure 3-16) was not able to bind the His tag in the t=0 incubation (i.e. when no TCEP was present). The intensity did increase following incubation with TCEP but this occurred in a single gradation providing a qualitatively identical response across all TCEP incubations. This suggests that at 0°C the reduction of the PDI tag is instantaneous (or at least takes less than 10 minutes) and therefore prolonged incubation is simply not required, which is fortuitous as this progressively inactivates the antibody. Confidence in the assertion that the reduction is selective to the PDI tag comes from the reference lane within the western which was reduced with dithiothreitol (Figure 3-15; lane 11 vs. Figure 3-16; lane 11). Considering the quantity of protein applied to the western was identical to the other lanes, the greater intensity of the band in the western suggests that once all disulphides are reduced and the histidine tag is even more accessible.
Figure 3-15. SDS-PAGE comparison of dAb reduction incubation time series at 0°C with 10mM TCEP. The dAb has an anticipated molecular weight of 16200 Da. Lane#1, SeeBluePlus2™; Lane#2, dAb without TCEP; Lane#3, dAb@10min; Lane#4, dAb@20min; Lane#5, dAb@40min; Lane#6, dAb@80min; Lane#7, dAb@160min; Lane#8, dAb@320min; Lane#9, SeeBluePlus2™; Lane#10, SeeBluePlus2™; Lane#11, dAb reduced with DTT. Slight contamination from lane#9 is observable in lane#8. Two lanes are used intentionally as a buffer between lane#11 and #8 to prevent DTT diffusion into lane#8 which may have affected the result.

Figure 3-16. Western blot of SDS-PAGE from Figure 3-16. Comparison of dAb reduction incubation time series at 0°C with 10mM TCEP. The dAb has an anticipated molecular weight of 16200 Da. Lane#1, SeeBluePlus2™; Lane#2, dAb without TCEP; Lane#3, dAb@10min; Lane#4, dAb@20min; Lane#5, dAb@40min; Lane#6, dAb@80min; Lane#7, dAb@160min; Lane#8, dAb@320min; Lane#9, SeeBluePlus2™; Lane#10, SeeBluePlus2™; Lane#11, dAb reduced with DTT. Slight contamination from lane#9 is observable in lane#8. Two lanes are used intentionally as a buffer between lane#11 and #8 to prevent DTT diffusion into lane#8 which may have affected the result.
3.4.2 Stoichiometric Conjugation of a Recombinant Antibody via a Representative Thiol Selective Chemistry

A thiol selective conjugation was attempted via maleimide chemistry using the heterofunctional cross linker illustrated in Figure 3-17. The mass of this molecular linker is 1450 Da and its addition can be monitored through protein mass shift in denaturing PAGE to determine the ratio of conjugation.

![Figure 3-17. Heterofunctional linker 94Å in length with biotin and maleimide functionalities. Molecular weight 1450 Da CAS#: 1334172-60-9](image)

Figure 3-18 and 3-19 illustrate a series of conjugations; three different temperatures across six different reaction conditions. The most interesting reaction condition is presented in Figure 3-18 across lanes 10 to 12 where both TCEP and thiol reactive linker are present (along with surfactant p20). This is interesting because at 0°C (Figure 3-19 lane 10) the reaction creates a conjugate whose mass shift corresponds to the addition of two linkers, as may be anticipated from the selective reduction and conjugation of the PDI tag. When the same reaction is undertaken at higher temperature (37°C, Figure 3-18 lane 11) a range of discrete products appear at masses correlating with the additions of 2, 4, 6 and 8 tethers. This suggests that at higher temperatures the thermal motion of the immunoglobulin fold allows the penetration of the reductant (and subsequently the maleimide linker) into its core resulting in the reduction/conjugation of internal structural disulphides. The presence of the surfactant in this reaction condition (p20, Figure 3-18 lane 12) serves to prevent the loss of the product though non-specific adsorptive processes or aggregation (as indicated by the reduced intensity of the bands when the surfactant is omitted Figure 3-18 lanes 6 to 8). Another notable result is that the unreduced protein is unchanged by extremes of temperature, as may be anticipated for this thermostable antibody, but as soon as TCEP is introduced into the reaction the stability of the protein at high temperature is compromised and the product is lost from solution. When only the maleimide tether is added, additions through non-selective side reactions do not result in discrete mass changes (i.e. identifiable banding patterns in the gel lane) rather they manifest as a broadening of the band (Figure 3-19 lanes 6 to 8). This suggests that the laddering seen in Figure 3-18 lane 10 is as a direct result of stoichiometric additions to four reduced disulphides.
Pegylated proteins do not migrate as anticipated in denaturing PAGE. The PEG functionality has two properties which lead to the retardation of electrophoretic movement through the gel; PEG binds the surfactant used to denature the protein and normalise protein mass:charge through hydrophobic exclusion, and the PEG polymer also increases the hydrodynamic radius of the protein but it does so disproportionately in relation to its relative mass. As a result the mass of the conjugated dAb is apparently increased massively but this is an artefact resulting from the choice of mass separation technique. As a result, the mass difference is empirical as the molecular weight standards have little relevance, but considering four bands are clearly visible it is a reasonable assumption that these equate to the pairwise addition of eight linkers in ~3kDa PEG increments (i.e. the mass of two linkers illustrated in Figure 3-17). This manifests as a serial mass addition with an approximate mass series (accounting for the disproportionate SDS-PAGE behaviour of PEG) of 25kDa, 44kDa, 71kDa and 106kDa equating to the addition of 2, 4, 6 and 8 linkers. The aberrant migration of the conjugated dAb through the gel is therefore thought to be an explainable anomaly.

Alternatively, it is possible that the four bands observed in Figure 3-18 lane 11 are in fact impurities which may also undergo conjugation in this reaction scheme. There are visible impurities in this purified dAb and so to determine whether this ladder does contain histidine tagged products a western blot was undertaken (Figure 3-20). From the western it is clear that the ladder of four bands is indeed His tagged and none of the impurities in the purified dAb preparation interfere with the assay. Interestingly the control/standards which were also present in this gel/western combination were completely invisible to the western immunoconjugate which repeats the observation in Figures 3-15 and 3-16 (that the His tag of unreduced dAb-PDI is occluded and therefore not available to His tag affinity reagents).
Figure 3-18. Optimisation of dAb-PDI reduction monitored via selective mass addition from maleimide functionalised linker conjugation (for controls see Figure 3-19). Samples were incubated at 0°C, 10mM TCEP for 3.5hr and subsequently with 15mM linker (Figure 3-17) for 45min. Stoichiometric mass additions to the dAb are annotated with green, yellow, red and blue arrows accounting for actual mass shifts of 3kDa, 6kDa, 9kDa and 12kDa but due to the aberrant behaviour of PEG in SDS-PAGE the apparent mass changes are anticipated to be 25kDa, 44kDa, 71kDa and 106kDa respectively.

Figure 3-19. Optimisation of dAb-PDI reduction monitored via selective mass addition from maleimide functionalised linker conjugation. Samples were incubated at 0°C, 10mM TCEP for 3.5hr and subsequently with 15mM linker (Figure 3-17) for 45min.
Figure 3-20. Western blot of histidine tagged proteins conjugated to thiol selective maleimide functionalised linker from Figure 3-18. Lane#1, SeeBluePlus2™; Lane#2, 0°C no additives; Lane#3, 37°C no additives; Lane#4, 0°C 10mM TCEP, 15mM linker 0.05% p20; Lane#5, 37°C 10mM TCEP, 15mM linker 0.05% p20. Stoichiometric mass additions to the dAb are annotated with green, yellow, red and blue arrows accounting for actual mass shifts of 3kDa, 6kDa, 9kDa and 12kDa but due to the aberrant behaviour of PEG in SDS-PAGE the apparent mass changes are predicted to be 25kDa, 44kDa, 71kDa and 106kDa respectively.
3.5 **Discussion**

**A model scFv system:** The scFv used as a model recombinant antibody was selected as it is well tolerated in *E. coli*. As demonstrated in denaturing PAGE comparison (Figure 3-8 relative to Figure 3-9) proportionally more of the HIS tagged product occurred as a soluble product rather than an insoluble aggregate. Though the product is not entirely soluble in this specific expression system (*lac* regulated in *E. coli* BL21 DE3) this is not unusual [92]. The fact that it was partially soluble makes it a useful reference system to compare the relative impact of C-terminal peptide fusions. It would have been possible to use this scFv to identify fusions which may either increase or decrease the solubility of the product.

**No fusion tags improve soluble scFv yield, relative to untagged product:** None of the tags tested increased the solubility of this scFv product. Considering the PDI peptide is an efficient substrate of DsbC there was a possibility that it may create a chaperone effect improving the rate of disulphide shuffling. No evidence suggests this to be the case.

**Less soluble tags are more toxic to the expression host:** A general trend is observed where the less soluble products have a greater impact on *E. coli* culture viability. The specific mechanisms underlying their insolubility are unknown, and may be unrelated. For example; the CYS product is the most toxic and contains an inherently reactive species known to cause issue within *E. coli* [77], and the SNAP product is a mammalian protein, which may be stable as a folded product, but *E. coli* lacks the posttranslational sophistication to cope with the quantity expressed in this system. Regardless of the mechanism underlying their relative insolubilities there is a qualitative correlation between product insolubility and culture viability. The outlier of the apparent trend in scFv solubility vs culture viability was the BMP tag. Its expression did not impact *E. coli* viability yet it generated a more insoluble product. This may be the result of aggregation inside the periplasm as opposed to within the cytoplasm. Wherever aggregation does occur it does not result in cellular toxicity to the same extent as the other aggregating materials tested (CYS and SNAP).

The generalised toxic effect of protein aggregation is well known though poorly understood [93] and remains a subject of broad research, from protein manufacturing to clinical disease (e.g. Parkinson’s and Alzheimer’s). Even when upregulated products are themselves native to *E. coli* they are known to aggregate when expressed in high yield [94]. This suggests there is a fundamental limitation in the capacity of this expression host to maintain order within its posttranslational systems. Indeed when the expressed product accounts for 30% of cellular protein the host machinery is thought to be so saturated that its viability is compromised [95]. So the saturation of cellular translation machinery leads to the fatigue of control systems and systemic collapse from the unrelenting stress induced. Considering high
yielding expression is such a traumatic process for the host anything which reduces the inherent solubility of the product is best avoided. The only tag to satisfy this aim was the PDI tag. It was tolerated during *E. coli* growth, and it did not reduce the soluble yield of the product.

**The PDI tag is a novel fusion peptide with beneficial properties:** The most remarkable attribute of the PDI tag is that it is a sequence which appears to be an efficient *in vivo* substrate for the posttranslational machinery of *E. coli*, specifically DsbC. This is perhaps not surprising considering it was originally designed as a synthetic substrate for kinetic studies of protein disulphide isomerases including DsbC [81]. However, it has never been applied as a fusion peptide and so this is a new observation. It is entirely possible that other cysteine containing peptides may generate similarly useful products (e.g. somatostatin fusion [96]) but it is interesting to note that the BMP peptide has potential to form a disulphide but the physical chemistry of this peptide makes it more prone to aggregate. So while the PDI tag is unlikely to be unique there remains scope to redesign the sequence as an affinity tag to impart additional functionality to it. This may be as a new affinity tag for *in vivo* chaperones or *in vitro* purification media.

The two thiols within the PDI tag may be exploited as site-specific functionalities for *in vitro* conjugation. Careful reduction of the recombinant antibody is required to avoid the reduction of native disulphides within the immunoglobulin fold. This was possible by optimising the reaction conditions using temperature to limit the thermal motion of the immunoglobulin fold and so limit the exposure of partially/fully buried disulphides within the Ig fold. This was successful though other reaction conditions may well have generated similar effect, such as the careful addition of TCEP to a stoichiometric ratio against the protein and the number of disulphides to be reduced [97]. Such an approach may be more time consuming but the products will require less clean up to remove unreacted TCEP before aberrant reduction occurs.

Once the dithiol within the PDI is reduced stoichiometric chemical conjugation is assumed to be occurring as the tag is anticipated to contain the most solvent exposed disulphides. In order to validate this unequivocally it would be necessary to undertake mass spectrometric peptide mapping of a conjugate like IAEDANS which is reported to ionise well [98]. The observation that the PEG modification was limited to two additions when reduction was undertaken at 0°C suggests that one disulphide is more solvent exposed than the others. It is a reasonable assumption that the most accessible disulphide may be the one present within the PDI tag.

This work has identified a generic tool for recombinant antibody engineering which should allow the simple and controllable integration of recombinant antibodies into biosensor interfaces. The benefits of orientated immobilisation have been widely reported and relate to the type of interface to which they are
integrated. The general principle that by controlling the point of attachment it is possible to reduce steric occlusion of the binding site is intuitive but the relative advantage depends on the type of interface the material is integrated into. For example, the benefit of orientation within three dimensional hydrogels is not as great as may be anticipated (typically it may double binding site activity). However, when applying antibodies to two dimensional surfaces their orientation has more profound implications.

Due to the nature of the disulphide within the PDI tag any reported method which uses the thiol functionalities within the hinge region of the Fc domain of antibodies, may be applied. The most significant advantages provided by the PDI tag, when applied to recombinant antibodies, is that it generates the same benefits while reducing the protracted processing required to convert whole antibody structures into F(ab)2 materials. In addition the minimisation of the antibody structure from the Fab’ to the single domain of the IgNARv or V\_H scaffolds should subsequently increase the surface density of biological recognition elements and reduce the quantity of extraneous protein immobilised within the sensing interface and with it the probability of protein mediated non-selective binding.

The PDI tag is also broadly applicable to other recombinant antibodies and exhibits the same superior solubility relative to the other tags tested, e.g. the BAP tag.
4. **Comparative Assessment of Covalent Passivation Chemistries and Surface Architectures for use as Affinity Biosensor Interfaces**

4.1 **Aim of this Chapter**

The work herein aims to fabricate an affinity biosensor interface to achieve two outcomes; to resist all non-selective biological interaction (i.e. protein adsorption to the interface itself), and accommodate functional biological recognition elements (i.e. antibodies immobilised upon the interface). The work is a novel synthesis of materials and methods reported in the literature. While the discrete components applied have been used explicitly for the functionality they reportedly provide, their combination herein is new.

Interfaces composed of adsorption resistant zwitterionic functionalities are presented in two-dimensional and three-dimensional architectures with oligoethylene glycol tethers for the conjugation of antibodies. The performance of the novel interfaces is quantitatively compared to literature standards (e.g. ethylene glycol and carboxymethyl dextran) specifically concerning; resistance to non-specific protein adsorption, antibody immobilisation density, and the activity of immobilised antibody.

4.2 **Covalent Interface Design, Production and Characterisation**

4.2.1 **Selected Synthetic Methods and Proposed Interfacial Architectures**

The successful integration of unnatural materials within biological systems remains a grand challenge. Many techniques have been developed to functionalise and characterise solid-liquid interfaces in order to explore their physical and chemical properties. These tools and techniques allow unparalleled control over interfacial chemistry. However, a critical comparison between interfacial architectures, and the functionalities they integrate, is absent.

Surface derivatisations may be categorised as either “graft to” or “graft from” depending on whether the deposited molecular arrangement results from chemical synthesis prior to, or in the course of, interface coupling. Of the “graft to” methods the most convenient for gold surface modification, and therefore widely reported, is the self-assembly of alkanethiol monolayers [126].

Alkanethiol self-assembly upon gold yields a two-dimensional molecular monolayer composed of aligned molecules orientated at 60 degrees from the plane of the gold surface (schematic shown in Figure 4-1). These monolayers coat the surface in high density due to lateral dispersion forces which stabilise a dense crystalline two-dimensional structure. Deposited monolayers may be single component or mixed by
simply mixing different alkanethiols in the deposition solvent. The exact proportion of mixed adsorbed species is unlikely to exactly match those in the deposition solvent but it is possible to form mixed SAMs easily and with an approximation between solution and surface molecular ratios [98].

In contrast to “graft to” methods the level of control afforded through “graft from” methods has been low (e.g. free radical polymerisation). A consequence of which has been reduced chemical and physical control of polymer interfaces, and the performance of the resulting interfaces have been compromised when applied within biological environments. For example, plasma polymerisation is an expedient method to coat many surfaces though the molecular control of physico-chemical properties requires extensive refinement and the precise physicochemical structures which result are difficult to characterise fully [127]. More conventional polymerisation methods maintain the fidelity of incorporated chemical entities but control of physical properties is limited by aberrant side reactions where propagating chains terminate prematurely (through combination, disproportionation or reaction with impurities). Advances in polymerisation techniques have resolved this issue and surface initiated “living-radical” polymerisation (e.g. ATRP, atom transfer radical polymerisation [128]) affords fine control of the deposited polymer which coats the interface allowing more control of the resulting chemical and physical properties.

The principle advantage of living-radical polymerisation (more accurately named reversibly-deactivated radical polymerization) is the preservation of propagating chains through the dilution of radical species, reducing the probability of their aberrant interaction. The ATRP method may therefore yield polymer chains in high density with prescribed physical properties. In such approaches the chemical identity of the polymer is dictated exclusively by the monomers incorporated and it’s physical behaviour dictated by
chain density and length. Monomers may be homogenous or mixed, deposited in either one synthesis or stratified through stepwise additions. The resulting interfaces are three-dimensional being composed of long “bottle brush” polymer chains perpendicular to the gold surface with pendant functionalities (schematic shown in Figure 4-2).

Figure 4-2. Schematic of an ATRP interface based upon oligoethylene glycol methacrylate. Polymerisation is initiated by specific halogen containing functionalities incorporated within the surface. Monomers (e.g. methacrylates) are sequentially added to the propagating chains. The resulting “bottle brush” polymers coat the surface in a self-limiting density dictated by steric occlusion from the propagating chains.

SAM and ATRP methods are employed here to allow controlled functionalisation of the SPR interface to incorporate chemical functionalities in two or three-dimensional arrangements respectively. Both methods should yield a contiguous surface coating to shield the underlying interface and, should appropriate functionalities be incorporated, they may prove inherently resistant to bio-fouling while simultaneously allowing the incorporation of biological recognition elements.

4.2.2 Selected Chemical Functionalities for the Passivation of Non-Selective Biological Interactions

The chemical functionalities which may provide the requisite function of binding water at the interface are numerous. A consensus view was presented by Kane et al. and increasingly divergent literature of tangential possibilities follows in-line with this: chemical functionalities that bind water present an enthalpic barrier to non-selective protein interaction [92]. All reported functionalities they may be easily
categorised according to how water interacts with them; through ionic or hydrogen bonding. The functionalities listed in Table 4-1 have been selected from these categories for comparison in SAM and/or ATRP deposition.

<table>
<thead>
<tr>
<th>Functionality</th>
<th>Solvation Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-ionic:</strong></td>
<td></td>
</tr>
<tr>
<td>Methoxy terminated oligoethylene glycol</td>
<td>H-bonding</td>
</tr>
<tr>
<td><img src="image" alt="Methoxy terminated oligoethylene glycol" /></td>
<td></td>
</tr>
<tr>
<td><strong>Ionic charge balanced pair:</strong></td>
<td></td>
</tr>
<tr>
<td>Sulphate with quaternary ammonium</td>
<td>Ionic solvation</td>
</tr>
<tr>
<td><img src="image" alt="Sulphate with quaternary ammonium" /></td>
<td></td>
</tr>
<tr>
<td><strong>Zwitterionic:</strong></td>
<td></td>
</tr>
<tr>
<td>Phosphorylcholine</td>
<td>Ionic solvation</td>
</tr>
<tr>
<td><img src="image" alt="Phosphorylcholine" /></td>
<td></td>
</tr>
</tbody>
</table>

Table 4-1. Table of pendant functional groups compatible with both SAM and ATRP deposition methods. The nature of “R” depends upon the deposition method employed being either alkanethiol or methacrylate.

It should be possible to create zwitterionic polymers composed of separate monomers of opposing charge which may be stoichiometrically combined to create a charge-balanced polymer product. A quantitative comparison of adsorption resistance afforded by such a surface, presented in either two- or three-dimensions, should be possible. Polyethylene glycol is a stock reference material for the construction of fouling resistant surfaces. Similarly zwitterionic phosphorylcholine monomers have been applied in fouling resistant materials and is employed here as an additional reference system.

4.2.3 Selected Conjugation Methods to Couple Biological Recognition Elements

In order to directly compare the ability of the prepared affinity interfaces to accommodate functional biological recognition elements, it is necessary to integrate functional groups into the surfaces to allow a
common derivatisation method to be applied. Ideally the same method should be applicable across all surfaces tested to minimise the variables and simplify comparison.

The use of nanoscale molecular tethers to couple biological recognition elements to the interface is known to improve the capture of cellular targets flowing past a solid surface (e.g. lymphocytes). Due to the size variation of potential biohazard materials it was desirable to use oligoethylene glycol as a molecular tether to couple biological recognition elements in all functionalised surfaces. PEG is known to be resistant to biofouling and so the fouling behaviour of the underlying interface should be unchanged.

The only commercially available oligoethylene glycol derivatives, compatible in both SAM and ATRP deposition methods, were hydroxyl terminated. It was hoped that these hydroxyls may be derivatised with a generic chemistry. However, the derivatisation method employed (100 mM DMAP, 100 mM DSC in DMF for 18 hours at room temperature [129]) destabilised the underlying OEG-SAM surface and subsequent modification was unsuccessful.

As a result of SAM instability it was necessary to minimise the manipulation of oligoethylene glycol containing SAM surfaces after alkanethiol deposition. Carboxyl functionalities were therefore incorporated within these SAM surfaces as part of the deposition process. Suitable carboxyls were commercially available and may be easily conjugated to proteins through conventional EDC/NHS coupling at the point of use [98]. This resolved the issue of SAM stability.

Further difficulty was experienced when protein conjugations were attempted on DSC activated ATRP surfaces (method as per Trmcic et al. [129]). Direct protein immobilisation failed. This may be through the instability inherent within the carbamate cross linker (i.e. hydrolysis or spontaneous devolution into carbon dioxide). Considering a maximum of 10% of incorporated monomers carry hydroxyls, these surfaces would be more sensitive to such issues relative to those reporting this method [129]. Alternatively this may pertain to steric confinement of the activated hydroxyls. Regardless, a heterofunctional linker composed of oligoethylene glycol flanked by amine and biotin functional groups, was therefore used to extend the tether and avoid the need for aqueous exposure of the DSC intermediate. The added biotin allowed affinity immobilisation of biological recognition elements on to the surface when streptavidin was used as an intermediary.

The immobilisation methods tested are summarised in Table 4-2 with their relative suitability annotated accordingly. As two immobilisation methods have been employed (EDC/NHS for SAMs and biotin/streptavidin for ATRP surfaces) direct comparison between surfaces would not be possible and so an additional SAM surface was made which presented a biotin in place of the carboxyl to allow comparison between SAM and ATRP surface architectures.
<table>
<thead>
<tr>
<th>Integrated Functionality</th>
<th>Antibody Immobilisation Route</th>
<th>Viability for BRE Coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM-OEG-Hydroxyl</td>
<td>DSC/DMAP 100mM in DMF for 18 hours</td>
<td>Unsuitable</td>
</tr>
<tr>
<td></td>
<td>Solvent manipulations of OEG-SAM interfaces destabilise the SAM</td>
<td></td>
</tr>
<tr>
<td>SAM-OEG-Carboxylic Acid</td>
<td>EDC/NHS activation of the carboxyl and direct immobilisation of the antibody</td>
<td>Suitable</td>
</tr>
<tr>
<td></td>
<td>Solvent exposure is short and occurs directly before binding analysis</td>
<td></td>
</tr>
<tr>
<td>SAM-OEG-Biotin</td>
<td>Streptavidin affinity for biotinylated antibody</td>
<td>Suitable</td>
</tr>
<tr>
<td></td>
<td>Required as a control system to compare ATRP surfaces</td>
<td></td>
</tr>
<tr>
<td>ATRP-OEG-Hydroxyl</td>
<td>DSC/DMAP 100mM in DMF for 18 hours followed by direct antibody immobilisation in aqueous phase</td>
<td>Unsuitable</td>
</tr>
<tr>
<td></td>
<td>The issue may relate to steric accessibility or the inherent instability of the carbamate/hydrolysis</td>
<td></td>
</tr>
<tr>
<td>ATRP-OEG-Biotin</td>
<td>Two stage derivatisation of the Hydroxyl functionality:</td>
<td>Suitable</td>
</tr>
<tr>
<td></td>
<td>i) DSC/DMAP 100mM in DMF for 18 Hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ii) NH$_2$-EG$_9$-Biotin 100mM in DMF for 18 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The addition of the amine-OEG-biotin extends the length of the OEG-tether within the ATRP brush and enables streptavidin binding</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. List of tested oligoethylene glycol derivatisation methods for immobilisation of protein biological recognition elements onto the sensing interfaces.

### 4.2.4 Interface Production and Characterisation

The commercially available Biacore “SIA” surface was used as a convenient SPR compatible consumable which is composed of 50 nm evaporated gold on a bonding layer of chromium upon BK7 glass. These chips

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were piranha cleaned immediately prior to use (Method 2.2.1). Cleaned SIA chips were used as a reference surface alongside two other commercially available proprietary Biacore chips known as “C1” and “CMS5” reported to present a carboxyl self-assembled monolayer and carboxymethyl dextran hydrogel respectively. These reference chips are summarised in Table 4-3.

<table>
<thead>
<tr>
<th>Surface Name</th>
<th>Integrated Functionalities</th>
<th>Fabrication Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIA</td>
<td>N/A</td>
<td>Biacore SIA chips</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Piranha treated before use</td>
</tr>
<tr>
<td>C1</td>
<td>HS-CX-COOH</td>
<td>Biacore C1 chips</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Used as received</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(design parameters are proprietary and therefore not known, e.g. length of the alkanethiol)</td>
</tr>
<tr>
<td>CMS5</td>
<td>Carboxymethyl dextran</td>
<td>Biacore CMS5 chips</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Used as received</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(design parameters are proprietary and therefore not known, e.g. the extent of carboxylation or molecular mass of the polysaccharide)</td>
</tr>
</tbody>
</table>

Table 4-3. Commercially sourced chips used in this work. Illustrations for indication only as the precise design and incorporated functionalities are proprietary.

After piranha cleaning, the gold SIA surfaces should be free from contamination. XPS analysis allows the determination of relative atomic abundance across the “clean” SIA surface (Table 4-4). As may be expected gold was the major species detected but other atomic species were found in the form of carbon, oxygen and sulphur which are thought to be from environmental contaminants. As soon as the cleaned surfaces are exposed to the environment they may readily accumulate contaminants (e.g. hydrocarbons).
Table 4-4. XPS quantitation of atomic species present on the piranha cleaned SIA gold surface used as a base from which to construct all affinity biosensor interfaces. Carbon species have been presented twice; once for relative total atomic count, and again as fractions of this count attributed to different molecular species. Gold may be anticipated to be the only species expected but environmental contaminants rapidly adsorb upon freshly cleaned gold.

AFM analysis of the “clean” SIA surface (Figure 4-3) shows a grain structure typical of evaporated gold [130]. The surface is not homogenous and nanoscale lumps (~150nm in diameter) are present (presumed to be gold). These surface imperfections facilitate visual correlation of surface features between related AFM images, as indicated by white circle annotations.

Designed SAM interfaces (Table 4-5) were fabricated by Ben Johnson (Leeds University) excluding the B-SAM and ATRPi-SAM which were fabricated by the author and Steve Edmondson (Loughborough
University) respectively. All surfaces were fabricated following methods prescribed by the author (methods 2.2.2 and 2.2.3 for SAM and ATRP respectively). SAM surfaces supplied by Leeds University were characterised immediately after production using XPS, ellipsometry and goniometry.

<table>
<thead>
<tr>
<th>Surface Name</th>
<th>Integrated Functionalities</th>
<th>Fabrication Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-SAM</td>
<td>100% methyl</td>
<td>Self-assembled onto Biacore SIA from 100% ethanol</td>
</tr>
<tr>
<td>OEG-SAM</td>
<td>90% HS-C_{11}-EG_{3}-OMe n=3</td>
<td>Self-assembled onto Biacore SIA from 100% ethanol</td>
</tr>
<tr>
<td></td>
<td>10% HS-C_{11}-EG_{6}-OCH_{2}-COOH n=6</td>
<td></td>
</tr>
<tr>
<td>CB/OEG-SAM</td>
<td>45% HS-C_{11}SO_3Na</td>
<td>Self-assembled onto Biacore SIA from 50:50 ethanol water</td>
</tr>
<tr>
<td></td>
<td>45% HS-C_{11}-NMe_3Cl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10% EG_6 carboxylic acid n=6</td>
<td></td>
</tr>
<tr>
<td>B-SAM</td>
<td>90% HS-C_{11}-EG_3-OMe n=3</td>
<td>Self-assembled onto Biacore SIA from 100% ethanol</td>
</tr>
<tr>
<td></td>
<td>10% HS-C_{11}-EG_6-Biotin n=6</td>
<td></td>
</tr>
<tr>
<td>ATRPi-SAM</td>
<td>100% HS-C_{11} OC(O)-IsoButyrate-Br</td>
<td>Self-assembled onto Biacore SIA from 100% ethanol</td>
</tr>
</tbody>
</table>

Table 4-5. Design of the SAM interfaces fabricated for this work.
The XPS data (Table 4-6) indicates that, relative to the cleaned SIA surface, the differences are attributable to the anticipated monolayers ascribed in Table 4-5. The B-SAM and ATRPi-SAM surfaces were not characterised through XPS as the equipment was not available.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Au</th>
<th>C</th>
<th>N</th>
<th>Cl</th>
<th>O</th>
<th>S</th>
<th>P</th>
<th>Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIA</td>
<td>63.6 ± 9.1</td>
<td>25.5 ± 5.1</td>
<td>-</td>
<td>-</td>
<td>9.3 ± 4.5</td>
<td>1.5 ± 1.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M-SAM</td>
<td>55.2*</td>
<td>34.9*</td>
<td>-</td>
<td>-</td>
<td>7.2*</td>
<td>2.6*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OEG-SAM</td>
<td>N/D*</td>
<td>75.9 ± 2.0</td>
<td>-</td>
<td>-</td>
<td>20.9 ± 1.5</td>
<td>3.1 ± 0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB/OEG-SAM</td>
<td>48.7 ± 0.7</td>
<td>39.6 ± 1.6</td>
<td>1.0 ± 0.3</td>
<td>-</td>
<td>5.4 ± 0.5</td>
<td>5.4 ± 1.5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4-6. XPS quantitation of atomic species present on the prepared self-assembled monolayers. Carbon species have been presented twice; once for relative total atomic count, and again as fractions of this count attributed to different molecular species. *Standard deviation of values for the M-SAM were not recorded. **OEG-SAM does not have a value for gold which was an omission in data processing. This has artificially inflated the relative values of the other species.

The thickness of the SAM layer (as determined by ellipsometry, Table 4-7) suggests that multilayers of aggregates were not present upon any of the surfaces. However, the values recorded do not agree with literature reported values. For example, OEG-SAM upon gold should be in the order of 16 Å thick [131, 132] but the value is 9.0 Å ± 1.0. It is possible the SIA gold surfaces present odd surface features (e.g. background roughness and nanoscale lumps) which may have artificially suppressed the calculated values. The variation in the values between SAM surfaces prepared herein is in proportion though, so they at least appear consistent within the same sample set. However, it is likely that these surfaces have a reduced density of alkanethiol chains as the ellipsometry data is also in poor agreement with literature reported values [131] and, more significantly, the goniometry data indicates significant hysteresis (difference) between advancing and receding water contact angles (i.e. 38 degrees relative to only 12 degrees as reported elsewhere [131]). Within this sample set the water contact angle data does indicate that the SAM surfaces interact differently with water, as may be expected, but it further confirms the quality of the SAM surfaces is not in-line with previously reported works using the same materials. The charge balanced SAM has the lowest contact angle of all the surfaces which suggests a strong interaction with water, but again, when compared to literature references of a very similar interface (i.e. containing the charged elements only, excluding the OEG tether) the values are higher than they should be [133]. The B-SAM and ATRPi-SAM surfaces were not characterised through ellipsometry or goniometry due to equipment availability.
<table>
<thead>
<tr>
<th>Surface</th>
<th>Ellipsometric Thickness (Å)</th>
<th>Advancing Water Contact Angle (°)</th>
<th>Receding Water Contact Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIA</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>M-SAM</td>
<td>7.9 ± 3.6</td>
<td>93 ± 4</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>OEG-SAM</td>
<td>9.0 ± 1.0</td>
<td>45 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>CB/OEG-SAM</td>
<td>7.0 ± 2.0</td>
<td>23 ± 3</td>
<td>Unmeasurable</td>
</tr>
</tbody>
</table>

Table 4.7. Ellipsometry and goniometry data for the SAM interfaces. Literature values for the OEG-SAM have been reported by Palegrosdemange et al. [131] and for CB/OEG-SAM by Holmlin et al. [133]. Neither ellipsometry or goniometry characterisation data agree entirely with these prior reports which suggests the surfaces made herein are not as finely controlled as initially intended.

The designed ATRP interfaces (Table 4-8) were fabricated by Steve Edmondson (Loughborough University) where preliminary characterisation was undertaken. More extensive verification was subsequently undertaken by Ben Johnson (Leeds University). The target thickness was 5 nm in the dry state with the expectation that this would swell upon hydration. The exact thickness change upon hydration will depend on a variety of factors, e.g. the density of the polymer chains or the stoichiometry of solvation. OEG-ATRP films, for example, have been reported to double in thickness. A thickness of 5 nm was chosen as it will locate immobilised binding elements close to the SPR interface which is required to sustain sensitivity in SPR biosensors. This is anticipated to be less of a concern for nanoscale antigens (e.g. toxins such as Ricin) but larger analytes (e.g. viruses) will benefit from being bound more closely to the interface. Any difference in polymer thickness is assumed not to impact the performance of the resistance barrier.
<table>
<thead>
<tr>
<th>Surface Name</th>
<th>Integrated Functionalities</th>
<th>Fabrication Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>OEG-ATRP</td>
<td>90% Poly(ethylene glycol) methyl ether methacrylate (n=4-5)</td>
<td>ARGET-ATRP onto ATRPi-SAM</td>
</tr>
<tr>
<td></td>
<td>10% Poly(ethylene glycol) methacrylate (n=6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td></td>
</tr>
<tr>
<td>CB/OEG-ATRP</td>
<td>45% 3-Sulfopropyl methacrylate potassium salt</td>
<td>ARGET-ATRP onto ATRPi-SAM</td>
</tr>
<tr>
<td></td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td></td>
</tr>
<tr>
<td></td>
<td>45% [2-(Methacryloyloxy)ethyl]trimethylammonium</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10% Poly(ethylene glycol) methacrylate (n=6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td></td>
</tr>
<tr>
<td>PC/OEG-ATRP</td>
<td>90% 2-methacryloyloxyethyl phosphorylcholine</td>
<td>ARGET-ATRP onto ATRPi-SAM</td>
</tr>
<tr>
<td></td>
<td><img src="image5" alt="Chemical Structure" /></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10% Poly(ethylene glycol) methacrylate (n=6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image6" alt="Chemical Structure" /></td>
<td></td>
</tr>
<tr>
<td>PC-ATRP</td>
<td>100% 2-methacryloyloxyethyl phosphorylcholine</td>
<td>ARGET-ATRP onto ATRPi-SAM</td>
</tr>
<tr>
<td></td>
<td><img src="image7" alt="Chemical Structure" /></td>
<td></td>
</tr>
</tbody>
</table>

Table 4-8. Design of the ATRP interfaces.
The XPS data (Table 4-9) indicates that, relative to the cleaned SIA surface, the differences in relative atomic abundance are attributable to the anticipated polymer layers ascribed in Table 4-8. The higher intensity for gold upon the OEG-ATRP surface suggests this surface is not as densely coated as the other ATRP surfaces.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Au</th>
<th>C</th>
<th>N</th>
<th>Cl</th>
<th>O</th>
<th>S</th>
<th>P</th>
<th>Na</th>
<th>C-C</th>
<th>C-O</th>
<th>COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIA</td>
<td>63.6 ± 9.1</td>
<td>25.5 ± 5.1</td>
<td>-</td>
<td>-</td>
<td>9.3 ± 4.5</td>
<td>1.5 ± 1.1</td>
<td>-</td>
<td>-</td>
<td>74.4 ± 9.6</td>
<td>19.3 ± 9.4</td>
<td>6.3 ± 1.1</td>
</tr>
<tr>
<td>OEG-ATRP</td>
<td>39.0 ± 3.0</td>
<td>44.0 ± 1.7</td>
<td>-</td>
<td>-</td>
<td>14.8 ± 1.5</td>
<td>2.1 ± 0.7</td>
<td>-</td>
<td>-</td>
<td>42.7 ± 2.9</td>
<td>48.6 ± 2.6</td>
<td>8.7 ± 0.6</td>
</tr>
<tr>
<td>CB/OEG-ATRP</td>
<td>15.7 ± 5.6</td>
<td>53.4 ± 2.4</td>
<td>2.5 ± 0.6</td>
<td>0.2 ± 0.2</td>
<td>21.2 ± 2.7</td>
<td>4.3 ± 0.5</td>
<td>-</td>
<td>-</td>
<td>54.7 ± 4.2</td>
<td>34.3 ± 3.7</td>
<td>11.0 ± 0.8</td>
</tr>
<tr>
<td>PC/OEG-ATRP</td>
<td>16.7 ± 2.5</td>
<td>50.1 ± 3.2</td>
<td>2.4 ± 0.7</td>
<td>0.7 ± 0.2</td>
<td>24.9 ± 2.1</td>
<td>1.9 ± 0.6</td>
<td>3.2 ± 1.0</td>
<td>0.1 ± 0.1</td>
<td>45.2 ± 3.7</td>
<td>45.1 ± 3.4</td>
<td>9.9 ± 0.5</td>
</tr>
<tr>
<td>PC-ATRP</td>
<td>15.7 ± 5.6</td>
<td>45.4 ± 5.3</td>
<td>3.0 ± 0.8</td>
<td>3.2 ± 1.3</td>
<td>27.1 ± 1.7</td>
<td>1.2 ± 0.9</td>
<td>3.6 ± 1.4</td>
<td>0.8 ± 1.3</td>
<td>43.8 ± 5.1</td>
<td>47.0 ± 5.0</td>
<td>9.2 ± 0.4</td>
</tr>
</tbody>
</table>

Table 4-9. XPS quantitation of atomic species present on the ATRP coated SIA gold biosensor interfaces. Carbon species been presented twice; once for relative total atomic count, and again as fractions of this count attributed to different molecular species.

Two methods were used to characterise the dry thicknesses of the ATRP polymer brushes; ellipsometry and atomic force microscopy (Table 4-10). Between the two methods employed the differences in measurement for CB/OEG-ATRP, PC/OEG-ATRP and PC-ATRP are insignificant (unpaired t-test p=0.342, p=0.066 and p=0.331 respectively). The only surface to show significant difference between measurement methods was the OEG-ATRP (unpaired t-test p=0.001).
Further investigation of the AFM images of the OEG-ATRP surfaces (Figure 4-4) reveals two types of surface feature which may be attributable to ATRP polymer deposition; a continuous layer (1.1 nm thick), and a series of lumps of uniform frequency (~1 feature per 10,000 nm$^2$ with varying diameter/height). This demonstrates a vulnerability of using ellipsometry in isolation when determining surface thickness – surface inhomogeneities are integrated into the average for the probed surface area giving rise to the gross error observed in Table 4-10.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Ellipsometry Thickness (nm)</th>
<th>AFM Thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OEG-ATRP</td>
<td>4.2 ± 0.4</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>CB/OEG-ATRP</td>
<td>5.0 ± 0.7</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td>PC/OEG-ATRP</td>
<td>5.0 ± 0.3</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>PC-ATRP</td>
<td>4.6 ± 1.5</td>
<td>6.0 ± 1.6</td>
</tr>
</tbody>
</table>

Table 4-10. Surface thickness of deposited polymers determined by ellipsometry and atomic force microscopy. n=9 three surfaces tested with three locations on each surface, variation of measurement is indicated by standard deviation.
Figure 4-4. AFM profile of the OEG-ATRP substrate (imaging relative height on the left and phase on the right). For convenience, three surface features within each image have been annotated with white circles to facilitate visual comparison between images. A) Profile of 4 µm² area. B) 5 µm² area image with scratch profile prepared using 300 nN sweeping force (applied as per method 2.2.4). Original line section, characterising the underlying SIA surface, is presented in 2.2.4 where a sweeping force of 3000 nN was required to clear the underlying gold. The sweeping force of 300 nN applied to clear both ATRP polymer and SAM coatings from this surface penetrates <0.1 nm into the underlying gold surface.
The lumps across the OEG-ATRP surface are distinct from those observed on the underlying SIA substrate (as seen in Figure 4-4). They are greater in number and are cleared from the surface by AFM force resistance profiling, whereas the SIA surface lumps can still be clearly seen within the cleared area (Figure 4-4B). However, there is a qualitative correlation between SIA surface lumps and polymer lumps. This suggests that polymer chains atop of the SIA lumps propagate more rapidly in the ARGET-ATRP deposition process. This is entirely speculative but monomer diffusion to the surface would be a rate limiting step and such surface protrusions would potentially increase local diffusion rates at these specific locations.

In comparison to the OEG-ATRP surface the CB/OEG-ATRP interface is more uniform (Figure 4-5). The ~5 nm coating across the surfaces is again peppered with lumps of polymer but they occur with reduced frequency. The PC/OEG-ATRP surface supports a continuous layer which is more uniform again relative to either the OEG-ATRP or the CB-ATRP surface (Figure 4-6). Surface roughness may be attributed to the underlying substrate. When a homologous polymer of the zwitterionic phosphorylcholine was deposited the surface is again peppered with many polymer lumps though a continuous layer is still uniform (Figure 4-7).
Figure 4-5. AFM profile of the CB/OEG-ATRP substrate (imaging relative height on the left and phase on the right). For convenience, three surface features within each image have been annotated with white circles to facilitate visual comparison between images. A) Profile of 5 µm² area. B) 5 µm² area image with scratch profile prepared using 300 nN sweeping force (applied as per method 2.2.4). Original line section, characterising the underlying SIA surface, is presented in 2.2.4 where a sweeping force of 3000 nN was required to clear the underlying gold. The sweeping force of 300 nN applied to clear both ATRP polymer and SAM coatings from this surface penetrates <0.1 nm into the underlying gold surface.
Figure 4-6. AFM profile of the PC/OEG-ATRP substrate (imaging relative height on the left and phase on the right). For convenience, three surface features within each image have been annotated with white circles to facilitate visual comparison between images. A) Profile of 4 µm² area. B) 5 µm² area image with scratch profile prepared using 300 nN sweeping force (applied as per method 2.2.4). Original line section, characterising the underlying SIA surface, is presented in 2.2.4 where a sweeping force of 3000 nN was required to clear the underlying gold. The sweeping force of 300 nN applied to clear both ATRP polymer and SAM coatings from this surface penetrates <0.1 nm into the underlying gold surface.
Figure 4.7. AFM profile of the PC-ATRP substrate (imaging relative height on the left and phase on the right). For convenience, three surface features within each image have been annotated with white circles to facilitate visual comparison between images. 

A) Profile of 4 µm² area. B) 5 µm² area image with scratch profile prepared using 300 nN sweeping force (applied as per method 2.2.4). Original line section, characterising the underlying SIA surface, is presented in 2.2.4 where a sweeping force of 3000 nN was required to clear the underlying gold. The sweeping force of 300 nN applied to clear both ATRP polymer and SAM coatings from this surface penetrates <0.1 nm into the underlying gold surface.
All surfaces support the selected chemical functionalities in the architecture afforded by the deposition method. The only significant deviation from the intended surface design is the OEG-ATRP surface which is significantly thinner than anticipated. There is also a variable distribution of polymer lumps across the different polymer types which may suggest polymerisation has not been as tightly controlled as intended as side reactions still appear to occur and to a variable extent between polymer types.

The mass density of polymer coatings may be compared through SPR and the absolute SPR response from the Biacore sensor from all the surfaces is presented in Figure 4-8 (data collected once surfaces were equilibrated in buffer and before subsequent adsorption testing reported in sections 4.3.2 and 4.3.3).

![Figure 4-8](image.png)

**Figure 4-8.** Comparison of adsorbed masses attributable to SAM/ATRP coatings upon each of the interfaces prepared. Values presented are absolute as they have been directly recovered from the Biacore 2000 SPR sensor as raw enumerated data. Surfaces were equilibrated in assay buffer (10 mM hepes buffered saline with 0.005% p20, unless otherwise stated) prior to protein adsorption screening and the absolute response of each equilibrated surface was recorded (n = 12 spread over three separate surfaces, 95% confidence intervals presented). As individual values, little meaning may be derived but variance between the values for each surface correlate directly with mass differences between surface coatings upon the gold SIA surface. On this basis, 1 unit difference between surfaces is equivalent to a mass difference of 1 pg.mm$^{-2}$ surface bound material. Surfaces are presented as follows; M-SAM without p20 surfactant [ ], piranha cleaned SIA gold surface [ ], commercial C1, OEG-SAM, CB/OEG-SAM, ATRPi-SAM, CMS, OEG-ATRP, CB/OEG-ATRP, PC/OEG-ATRP, PC-ATRP. There is a clear difference between the two-dimensional and three-dimensional surfaces (denoted as broken and solid coloured bars respectively). For example, the CMS surface is three-dimensional and carries upon it carboxymethyl dextran at a mass density of 1.4 ng.mm$^{-2}$ (calculated relative to the C1 surface from the same commercial supplier, and so it is assumed the underlying SAM is of similar mass between these two surfaces). Of the three-dimensional surfaces CB/OEG-ATRP has the greatest mass of material present.
A broad difference between two- and three-dimensional interfaces is clear. There are also differences between surfaces within the two categories, notably for C1 and CB/OEG-ATRP surfaces. A complication within this data set is that the buffer used to equilibrate the surfaces was not identical across all surfaces. The M-SAM surface was equilibrated without surfactant p20 where all other surfaces were exposed to buffer containing the surfactant (justified fully in section 4.6.2). The refractive index of the bare gold surface is insignificantly different to the M-SAM (unpaired t test p=0.108) which is not possible considering the underlying surface of each is the same and one carries an alkanethiol monolayer where the other is bare. PEG is known to have an affinity for gold and through the surfactants dendritic sorbitan structure it is possible that this surfactant binds to the gold surface to an extent. Any difference in refractive index between chips may therefore be attributable to two indistinguishable factors; the mass of material forming the covalent interface, and the mass of surfactant non-covalently associated with the interface.

It is unlikely that the extent of p20 adsorption to the ATRP brushes would vary with functionality. Any adsorption of p20 within these surfaces would likely be to the methacrylate chain, and therefore correlate with chain density. The significantly higher refractive index of the CB/OEG-ATRP surface (e.g. unpaired t test relative to PC/OEG-ATRP p<0.001) may therefore indicate a greater chain density upon this surface while all other ATRP surfaces were insignificantly different (one-way ANOVA p=0.228 for PC/OEG-ATRP, PC-ATRP and OEG-ATRP surfaces).

Surfactant adsorption across the two-dimensional surfaces is likely to be less predictable as it may relate to the frequency of imperfections across the SAM which may relate to the relative densities of specific functionalities coating the surface. However, considering the linear correlation between refractive index and the average molecular mass of the SAMs deposited (Figure 4-9) surfactant adsorption to the hydrophilic SAMs is thought to be low, if it is present at all. Due to the higher refractive index observed upon the C1 surface it is probable that this surface is composed of a longer alkanethiol SAM than indicated in Table 4-3.
4.3 **Comparison of Antibody Immobilisation Densities and their Resultant Binding Activities within the Interfaces**

The most critical performance parameter of an affinity biosensor interface is the ability to accommodate functional recognition elements. Through the methods developed in section 4.2.3 it was possible to immobilise recognition elements to all interfaces. Three immobilisation strategies were employed; physical adsorption, amine coupling and biotin affinity. The densities of recognition elements immobilised are listed in Table 4-11.
Antibody was successfully immobilised onto all sensor interfaces. Reference systems for comparison of test surfaces are SIA, CM5 and B-SAM. Antibody adsorption onto gold yielded modest density with high variability. In contrast, immobilisation by amine conjugation (to CM5) or streptavidin affinity (to B-SAM), demonstrated that these strategies may increase the densities achieved while reducing inter-chip variability. The CM5 interface is a three-dimensional hydrogel enabling antibody to be immobilised within a three-dimensional polymer framework. In contrast the B-SAM is a planar two-dimensional interface enabling planar, two-dimensional immobilisation. This system indicates the maximal density which may be achieved on a planar interface (as this affinity immobilisation is not limited by hydrolysis as is EDC/NHS and so the only limit of immobilisation density is steric occlusion across the two-dimensional plane).

None of the immobilisations on the test surfaces exceeded a monolayer which suggests that all the interfaces present a planar, contiguous barrier. The ionic interfaces interacted variably with IgG. Any adsorbed IgG would most likely have desorbed from the CB/OEG-SAM during chip equilibration and conditioning (e.g. exposure to glycine buffer pH 2.5). In contrast the CB/OEG-ATRP surface was able to accommodate a high density of antibody though variability between immobilisations was high. Both non-
ionic surfaces (i.e. OEG-SAM and OEG-ATRP) were more reproducible and the densities achieved were in excess of those achieved through physical adsorption. OEG interfaces, whether deposited via SAM or ATRP, immobilise IgG in high density.

The percentage activities of immobilised rabbit polyclonal antibodies listed in Table 4-11 are presented in Table 4-12.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Immobilisation method</th>
<th>Antibody Binding Site Activity (%)</th>
<th>Inter Chip Variability (CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIA</td>
<td>Adsorption</td>
<td>29.9 ± 4.5</td>
<td>15.1</td>
</tr>
<tr>
<td>CM5</td>
<td>Amine</td>
<td>23.3 ± 1.8</td>
<td>7.7</td>
</tr>
<tr>
<td>OEG-SAM</td>
<td>Amine</td>
<td>24.4 ± 1.5</td>
<td>6.2</td>
</tr>
<tr>
<td>CB/OEG-SAM</td>
<td>Amine</td>
<td>16.8 ± 5.1</td>
<td>30.4</td>
</tr>
<tr>
<td>B-SAM</td>
<td>Streptavidin</td>
<td>12.4 ± 1.6</td>
<td>12.9</td>
</tr>
<tr>
<td>OEG-ATRP</td>
<td>Streptavidin</td>
<td>6.9 ± 0.9</td>
<td>13.0</td>
</tr>
<tr>
<td>CB/OEG-ATRP</td>
<td>Streptavidin</td>
<td>6.1 ± 2.7</td>
<td>44.3</td>
</tr>
</tbody>
</table>

Table 4-12. Comparison of binding activity of rabbit polyclonal antibody immobilised upon the interfaces listed. Binding activity is calculated as the number of antigen molecules specifically bound by the surface after exposure to antigen (hen egg ovalbumin) at a concentration of 100 μg.ml⁻¹, divided by the total number of binding sites immobilised (i.e. two sites per antibody molecule). The fractional activity is expressed as a percentage. n = 9 distributed over three surfaces, 95% confidence intervals are indicated along with an associated Coefficient of Variation.

All interfaces were shown to support functional antibody to some extent. Adsorbed antibody on the SIA surface demonstrated the highest activity of 29.9%. This was significantly better than amine coupled antibody on both the OEG-SAM (p=0.003 unpaired t test) and CM5 (p=0.001 unpaired t test). These amine coupled surfaces had insignificantly different activities of ~24% (p=0.178 unpaired t test). However, activity was significantly reduced when antibody was amine immobilised on the CB/OEG-SAM (p=0.002 unpaired t test). This suggests that, relative to the other amine immobilised interfaces, on this interface immobilised antibody is less active. Relative to the OEG-SAM the density is lower therefore steric occlusion is unlikely to be the cause and the lower activity may relate to poor orientation or denaturation.
Comparison between the B-SAM and OEG-SAM clearly indicates that antibody biotinylation, and high density streptavidin affinity immobilisation, has inactivated half of the antibody binding sites immobilised. Biotinylation was undertaken via amine conjugation which may have chemically inactivated a proportion of the binding sites. However, it is also possible that this reduced activity may be a function of steric occlusion as the mass of protein adsorbed is significantly higher than the OEG-SAM (p<0.001 unpaired t test). Interestingly the activity of the biotinylated antibody is further reduced or impeded when immobilised on the ATRP surfaces. Relative to the B-SAM immobilisation density is reduced and so the lower activity must relate to a function of the interface itself, i.e. binding is unlikely to be inhibited by the sheer quantity of antibody immobilised which may be the case with the B-SAM. The OEG tethers used to immobilise the antibody on the ATRP surfaces are longer than the B-SAM. The ATRP tethers are equivalent to 15 ethylene oxide repeats as opposed to 6 on the B-SAM. This equates to a linear tether length of 5.2 and 2.1 nm respectively. It is interesting to note that the activity of antibody on both ATRP surfaces was equivalent (p=0.412 unpaired t test). As the reduction in activity is common to both surfaces, despite the difference in polymer content, it may well relate to the extended length of the tether which is common to them both. However, it may also relate to other common elements between the surfaces such as aberrant interactions with the methacrylate backbone.

The data used to calculate density and activity in Tables 4-11 and 4-12 has been plotted as a scatter plot (Figure 4-10). Nine data points are presented for each surface type generated from three different surfaces. As discussed above the only surface supporting a three dimensional distribution of antibody is the CM5 interface. All other tested surfaces accommodate a mass of antibody equivalent to a monolayer or less.
Figure 4-10. Re-presentation of immobilised antibody density plotted against percentage activity. A surface mass commensurate with a monolayer of antibody is indicated by a vertical dashed line. Each point represents a separate interface upon which multiple activity tests were undertaken in series (10 mM potassium hydroxide used to regenerate the binding interface and strip the antigen from the antibody). Variation of binding activity for each interface tested is indicated as a standard deviation where n = 5.

4.4 Comparison of Non-Selective Interactions Between Buffered Solutions of Model Proteins and the Interfaces

4.4.1 Selection and Characterisation of Model Proteins for Adsorption Comparison

Model adsorption systems were used to compare relative non-specific protein adsorption across the surfaces: these are listed in Table 4-13. These were selected as they have been extensively reported in the literature and may therefore allow comparison with reference studies. All non-specific binding tests were undertaken on interfaces without antibodies immobilised to allow characterisation of the underlying interface.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass (g.mol⁻¹)</th>
<th>Isoelectric Point (pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep serum</td>
<td>Heterogeneous</td>
<td>Heterogeneous</td>
</tr>
<tr>
<td>Hen Egg Lysozyme</td>
<td>14,400</td>
<td>11.0</td>
</tr>
<tr>
<td>Rabbit Immunoglobulin Gamma</td>
<td>150,000</td>
<td>Variable</td>
</tr>
<tr>
<td>Bovine Fibrinogen</td>
<td>340,000</td>
<td>5.5</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>66,500</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Table 4-13.* Table comparing the physical properties of the protein panel selected to screen the non-specific binding behaviour of the interfaces developed. These have been chosen to cover a range of charges and sizes. Sheep serum is included as a heterogeneous mixture with varying components in varying proportions, undefined but valuable as a complex mixture.

The materials were commercially sourced and their purities were qualitatively compared through reducing SDS-PAGE (*Figure 4-11*). Disulphides within the proteins are reduced due to the addition of DTT and any inter-subunit disulphides would have been broken, the result being each subunit travels through the gel independently (e.g. the heavy and light chains of the IgG or the various subunits of fibrinogen).
Figure 4-11. Reducing SDS-PAGE gel indicating the purity and size of the proteins used in the adsorption screening panel. Lanes 1 and 7 are molecular weight markers (SeeBlue Plus2), lane 2 sheep serum, lane 3 lysozyme, lane 4 rabbit polyclonal IgG, lane 5 fibrinogen and lane 6 bovine serum albumin.

The correlation between anticipated molecular weight (Table 4-11) and observed molecular weight (Figure 4-11) are consistent apart from BSA which contains significant quantities of at least two other products of greater apparent mass (e.g. 200 kDa and 100 kDa). These higher molecular weight proteins are thought to be oligomers of BSA resulting from commercial purification processes.

4.4.2 Characterisation of Model Protein Adsorption at a Model Hydrophobic Interface

In order to understand the efficiency of fouling resistance afforded by the different surface treatments it is necessary to understand the extent of non-selective binding which may be expected when the model proteins (listed in Table 4-11) interact with surfaces which are not engineered to resist protein fouling. Only by doing this is it possible to determine the relative advantage of any surface modification strategy or
class of integrated functionalities. It is necessary therefore to characterise the specific fouling system used herein to account for the potential variability of the proteins and their adsorption processes within the specific experimental system used (e.g. buffer, salts etc.). In order to experimentally control for such inevitable local experimental variables the Whitesides group used a methyl terminated self-assembled monolayer as an adsorption standard which allowed them to empirically compare different surface functionalities [134]. This has been emulated here, the surface denoted herein as the M-SAM (methyl self-assembled monolayer). This method allows convenient comparison between surfaces through the calculation of percentages of monolayers formed relative to this protein fouling interface.

In this work a surface plasmon resonance sensor (Biacore 2000) was used to control the exposure of M-SAM surfaces to a series of protein injections and quantify the protein adsorbed (Method 2.2.5). A representative series of sensograms is shown in Figure 4-12. Data points were collected before, during and after each injection (as indicated with arrows in Figure 4-12). At point “A” the total refractive index change is the sum of two protein responses; the bulk response of the injected volume (for 1 mg.ml\(^{-1}\) protein solution this equals 200 resonance units, or 200 x 10\(^{-6}\) refractive index units), and any protein interacting with the surface (reversibly or otherwise). At point “B” the injection has terminated and the surface re-equilibrated in running buffer. The bulk refractive index response is therefore removed and the value observed is solely attributable to persistent surface adsorbed protein. Point “C” is the refractive index of the interface following buffer equilibration. Referencing “A” and “B” to this point (as illustrated, the responses have been normalised to this point) allows the refractive index change attributable to protein to be quantitatively determined for persistently adsorbed protein (point “B”), and empirically indicated for weakly interacting protein (point “A”).

Observed protein adsorption may be considered as either of higher or lower affinity. In a practical sense this means that the interaction is either; immediately reversible and may be washed easily from the surface with buffer, or irreversible remaining bound to the surface after washing with buffer. It is most convenient to measure irreversible adsorption as this may be calculated simply by subtracting the pre-exposure baseline from the final measurement after exposure. While such tests are widely published, and undeniably pragmatic, they ignore reversible binding which may be equally as inconvenient for real-time biosensors as irreversible adsorption. To be truly fouling resistant the sensor interface must not change the properties of the system it is interrogating: the inadvertent concentration of protein at, or in close proximity to, the interface is still being driven by the energetics of the interface [135]. Using SPR, reversible adsorption may only be qualitatively compared as the in situ binding response (indicated in Figure 4-12) is composed of masses of proteins attributable to; bulk protein within the solvent, protein irreversibly adsorbed to the surface, and protein which is reversibly coordinated to the surface. It is not possible to deconvolute this data as adsorbed protein effectively screens adsorption from other proteins.
and so facile subtraction of irreversibly adsorbed protein is an unsound approach to calculate reversibly adsorbed protein. However, qualitative comparison of in situ binding responses may still be informative. Twelve separate surface areas were exposed across three separate chips. The cleaning process used to strip adsorbed protein was not 100% efficient. This has introduced a degree of variability into the data though average adsorption for each model protein upon the M-SAM (with 95% confidence intervals) shows clear differences between proteins (tabulated in Table 4-14).

<table>
<thead>
<tr>
<th>Protein (1 mg/ml)</th>
<th>Adsorbed Protein (pg.mm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep serum</td>
<td>1643 ± 175</td>
</tr>
<tr>
<td>Hen Egg Lysozyme</td>
<td>1441 ± 273</td>
</tr>
<tr>
<td>Rabbit IgG (polyclonal)</td>
<td>2968 ± 503</td>
</tr>
<tr>
<td>Bovine Fibrinogen</td>
<td>3749 ± 248</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>1159 ± 157</td>
</tr>
</tbody>
</table>

Table 4-14. Extent of stable non-specifically adsorbed protein (as defined in Figure 4-12) upon the M-SAM surface (n = 12 over three separate surfaces, 95% confidence intervals presented).

Irreversible protein adsorption on the M-SAM surface should theoretically be correlated via a power relationship to molecular mass (which correlates with protein hydrodynamic radius). Adsorption data for the homogenous model proteins listed in Table 4-14 has been plotted in Figure 4-13 and is described by the anticipated power relationship. In agreement with literature reports the densities achieved are significantly reduced relative to an idealised hexagonal arrangement (i.e. ~50% thereof) [136]. This is thought to result from random sequential adsorption which may progressively screen the surface with adsorbed material. BSA is an outlier from this trend and does not adsorb at a related proportional density. This cannot be explained by the impurities detected through SDS-PAGE (Figure 4-11) as these were of higher molecular mass and would therefore increase the density of material, not decrease it. It is possible that BSA is a more flexible structure relative to the other proteins and so it may spread upon the M-SAM surface once it has adsorbed, though this is speculative.
Figure 4-13. Non-selective adsorption of model proteins upon the M-SAM surface (in the absence of surfactant p20). For each point n = 12 over three separate surfaces with 95% confidence intervals indicated. Proteins plotted increase in mass (lowest to highest); lysozyme, bovine serum albumin; IgG and fibrinogen. The plotted formula attempts to correlate adsorbed mass (determined via SPR) with protein molecular mass via a power function.

The method used above omitted surfactant from all buffers (apart from the SDS used to clean the chip between injections). This was necessary to ensure the surface behaviour of the hydrophobic SAM was not inadvertently modified. A consequence of the omission of surfactant (such as p20 as advised by the manufacturer of the SPR system) was protein aggregation and some of the surface tests failed as a result. Such failure was sporadically observed but only for hen egg lysozyme and IgG, and for only two and three data points respectively across twelve data points. This data was necessarily discarded as it was unusable and p20 surfactant was added to subsequent adsorption tests to prevent aggregation.

4.4.3 Comparative Testing of Model Protein Adsorption Across the Interfaces: Extent of Adsorbed Protein Mass Irreversibly and Reversibly Associated

The extent of irreversibly adsorbed protein mass (as defined in Figure 4-12) upon all tested surfaces is presented in Table 4-15.
<table>
<thead>
<tr>
<th>Surface</th>
<th>Lysozyme (pg.mm$^{-2}$)</th>
<th>Fibrinogen (pg.mm$^{-2}$)</th>
<th>Sheep Serum (pg.mm$^{-2}$)</th>
<th>Rabbit IgG (pg.mm$^{-2}$)</th>
<th>BSA (pg.mm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-SAM - p20</td>
<td>1441 ± 273</td>
<td>3749 ± 248</td>
<td>1643 ± 175</td>
<td>2968 ± 503</td>
<td>1159 ± 157</td>
</tr>
<tr>
<td>SIA</td>
<td>575 ± 287</td>
<td>807 ± 505</td>
<td>471 ± 256</td>
<td>703 ± 325</td>
<td>156 ± 149</td>
</tr>
<tr>
<td>OEG-SAM</td>
<td>46 ± 27</td>
<td>89 ± 66</td>
<td>215 ± 22</td>
<td>257 ± 23</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>CB/OEG-SAM</td>
<td>88 ± 51</td>
<td>459 ± 193</td>
<td>906 ± 104</td>
<td>1604 ± 218</td>
<td>53 ± 18</td>
</tr>
<tr>
<td>ATRP-SAM</td>
<td>189 ± 48</td>
<td>241 ± 85</td>
<td>564 ± 96</td>
<td>258 ± 45</td>
<td>74 ± 41</td>
</tr>
<tr>
<td>OEG-ATRP</td>
<td>141 ± 40</td>
<td>24 ± 4</td>
<td>34 ± 7</td>
<td>27 ± 5</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>CB/OEG-ATRP</td>
<td>101 ± 47</td>
<td>46 ± 7</td>
<td>208 ± 20</td>
<td>88 ± 12</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>PC/OEG-ATRP</td>
<td>100 ± 67</td>
<td>27 ± 10</td>
<td>154 ± 29</td>
<td>104 ± 30</td>
<td>21 ± 23</td>
</tr>
<tr>
<td>PC-ATRP</td>
<td>248 ± 89</td>
<td>64 ± 28</td>
<td>181 ± 48</td>
<td>93 ± 26</td>
<td>73 ± 33</td>
</tr>
<tr>
<td>C1</td>
<td>21 ± 9</td>
<td>48 ± 10</td>
<td>241 ± 29</td>
<td>138 ± 31</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>CM5</td>
<td>18 ± 8</td>
<td>6 ± 1</td>
<td>46 ± 4</td>
<td>36 ± 3</td>
<td>5 ± 3</td>
</tr>
</tbody>
</table>

Table 4-15. Extent of stable non-specifically adsorbed protein (as defined in Figure 4-12) upon the surfaces listed (and defined in Tables 4-3, 4-5 and 4-8). Listed values are calculated masses of adsorbed protein per unit surface area (n = 12 over three separate surfaces, 95% confidence intervals presented).
Relative to the M-SAM all surface treatments reduce the extent of protein fouling. Adsorption to the gold SIA surface was less reproducible than any of the other surfaces (indicated by broad 95% confidence intervals). SDS was found to be less efficient in cleaning this surface for repeated surface testing. As all other surfaces generated more defined confidence intervals, relative to the gold surfaces, it is therefore reasonable to assert that all surface coatings tested did not delaminate from the gold and they were effectively cleaned with SDS treatment between protein exposures. Another broad observation from Table 4-15 is that BSA appears not to adsorb strongly to any of the derivatised surfaces. Relative to the M-SAM the general magnitude of BSA adsorption across all modified surfaces is low. Further comparative interpretation of this data required calculation of relative monolayer formation and is presented and discussed in section 4.3.4.

Lower affinity, reversible adsorption may also provide insight to surface fouling behaviour. Total mass responses (defined in Figure 4-12) may be used to indicate the relative extent of reversible surface interaction (Figure 4-14). This data does not allow quantitative comparison though does allow qualitative indication. Most of the adsorption indicated in Figure 4-14 may be explained as irreversible adsorption (i.e. irreversible adsorption broadly accounts for the measured responses in Table 4-15). There are, however, two points of specific interest; lysozyme adsorption to CM5, and the apparent resistance of BSA adsorption by all surfaces.
Figure 4-14. SPR derived, peak protein adsorption values observed during exposure of interfaces to the non-specific protein panel (see Figure 4-12 binding curve point “A” for an example and explanation of the measurement). The dashed line indicates the SPR response (i.e. bulk refractive index change) attributable to protein in solution at a concentration of 1 mg.ml⁻¹ (as determined in method 2.2.6). Anything observed at this level can in no way be attributed to any surface interaction, but anything above this level is clearly interacting with the surface as the protein is in effect being concentrated at the surface.

Surfaces are presented as follows; OEG-SAM, CB/OEG-SAM, commercial C1, ATRP-SAM, OEG-ATRP, CB/OEG-ATRP, PC/OEG-ATRP, PC-ATRP, CMS. BSA does not interact appreciably with any of the surfaces whereas lysozyme demonstrates a clear preference to interact with the CMS surface. IgG is prone to fouling upon two-dimensional surfaces.

As a single protein concentration of 1 mg.ml⁻¹ was used for all protein exposures the bulk refractive index during the exposure should be similar across all the proteins tested. This is the case for all CMS exposures except for lysozyme which, during the time of the exposure, exhibits significant retention within the carboxymethyl hydrogel. This is attributable to the isoelectric point of this protein (Table 4-13) and the complementary charge interaction with the carboxylates within the dextran hydrogel. This is trivial but does indicate that if any significant level of reversible adsorption should occur upon, or within, any of the other surfaces then it may be detectable. This does not occur.

An important indication from Figure 4-14 is that the bulk refractive response to BSA is relatively uniform across the all surfaces. As BSA was found not to bind to any great extent across the modified surfaces.
(Table 4-15) and the near uniformity of their optical response indicates that biosensor sensitivity is broadly uniform across the surfaces tested. In the following sections the quantitative interpretation of stably adsorbed binding resistance is predicated on the assumption that all interfaces were equally sensitive to protein adsorption. The magnitude of SPR response is distance dependant and, by design, the two- and three-dimensional interfaces are of different thickness. However, the BSA data indicates that this difference has not detectably affected assay sensitivity. This is an important validation underpinning all subsequent interpretation.

4.4.4 Comparative Testing of Model Protein Adsorption Across the Interfaces: Irreversible Adsorption as a Percentage Monolayer

The adsorption upon the M-SAM surface is assumed to be as close to a monolayer as may be achieved through random adsorption [137]. As such, stably adsorbed protein upon the two-dimensional surfaces is re-presented in Table 4-16 as fractional values (expressed as a percentage) of the adsorption observed upon the M-SAM surface.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Lysozyme (% monolayer)</th>
<th>Fibrinogen (% monolayer)</th>
<th>Sheep Serum (% monolayer)</th>
<th>Rabbit IgG (% monolayer)</th>
<th>BSA (% monolayer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-SAM - p20</td>
<td>100.0 ± 18.9</td>
<td>100.0 ± 6.6</td>
<td>100.0 ± 10.7</td>
<td>100.0 ± 17.0</td>
<td>100.0 ± 13.6</td>
</tr>
<tr>
<td>SIA</td>
<td>39.3 ± 19.9</td>
<td>21.5 ± 13.5</td>
<td>28.7 ± 15.6</td>
<td>23.7 ± 11.0</td>
<td>13.5 ± 13.0</td>
</tr>
<tr>
<td>OEG-SAM</td>
<td>3.2 ± 1.9</td>
<td>2.4 ± 1.8</td>
<td>13.1 ± 1.3</td>
<td>8.7 ± 0.8</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>CB/OEG-SAM</td>
<td>6.1 ± 3.6</td>
<td>12.2 ± 5.2</td>
<td>55.1 ± 6.3</td>
<td>54.0 ± 7.3</td>
<td>4.6 ± 1.6</td>
</tr>
<tr>
<td>C1</td>
<td>1.4 ± 0.6</td>
<td>1.3 ± 0.3</td>
<td>14.7 ± 1.8</td>
<td>4.6 ± 1.0</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>ATRPi-SAM</td>
<td>13.1 ± 3.3</td>
<td>6.4 ± 2.3</td>
<td>34.4 ± 5.8</td>
<td>8.7 ± 1.5</td>
<td>6.4 ± 3.6</td>
</tr>
</tbody>
</table>

Table 4-16. Stably adsorbed model proteins listed in Table 4-13 on surfaces listed. Listed values are expressed as percentages of adsorbed protein observed upon the M-SAM surface. Error has been calculated as the 95% confidence interval where n = 12.

The data in Table 4-16 is re-presented graphically in Figure 4-15. The most resistant surfaces were the C1 and OEG-SAM which repelled large and small proteins alike, though IgG and serum were found to non-
specifically bind to a greater extent. The CB/OEG-SAM was found to adsorb all proteins, especially IgG and serum. The ATRPi-SAM was found to be relatively non-fouling, which was unexpected as it is not a hydrophilic interface (with a reported contact angle of >80° [138]).

![Graphical comparison of protein adsorption upon all two-dimensional interfaces listed in Table 4-16; M-SAM without p20 surfactant, piranha cleaned SIA gold surface, commercial C1, CB/OEG-SAM, OEG-SAM, ATRPi-SAM. Unless otherwise indicated all surfaces were tested in the presence of p20 surfactant at 0.005% mass. volume. 95% confidence interval presented n = 12 with samples spread over three surfaces.](image)

The relative extent of SAM adsorption was replotted using a scatter plot on a log-log scale (Figure 4-16). Consistent fractions of monolayers appear to adsorb to the two-dimensional interfaces, though the significance of this correlation is low. IgG appears to deviate from this apparent trend across all the surfaces. Excluding IgG the relative percentage monolayers are ~1% for C1, ~10% for CB/OEG-SAM and between 1% and 10% for OEG-SAM.
Figure 4-16. Non-selective adsorption of model proteins upon two-dimensional SAM surfaces. For each point n = 12 over three separate surfaces, with 95% confidence intervals indicated. Proteins plotted increase in mass (lowest to highest): lysozyme, bovine serum albumin; IgG and fibrinogen. The plotted formula attempts to correlate the percentage of a monolayer adsorbed with protein molecular mass using a power function. There is no correlation between the extent of fouling and the molecular mass of the protein upon these two-dimensional surfaces. Empirically, however, adsorption does appear to be relatively uniform as a consistent monolayer fraction, principally observed with the C1 interface where adsorption occurs reproducibly as 1% of a monolayer.

The comparative percentages of irreversible protein adsorption across the three-dimensional ATRP surfaces are presented in Table 4-17 and graphically in Figure 4-17. As a control surface the ATRPi-SAM is
included here again for comparison as it is the surface from which three-dimensional ATRP polymers were propagated. This SAM is therefore an understory beneath the ATRP polymers: should the deposited polymers increase or decrease their propensity for protein adsorption it will be relative to this SAM.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Lysozyme (% monolayer)</th>
<th>Fibrinogen (% monolayer)</th>
<th>Sheep Serum (% monolayer)</th>
<th>Rabbit IgG (% monolayer)</th>
<th>BSA (% monolayer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-SAM - p20</td>
<td>100.0 ± 18.9</td>
<td>100.0 ± 6.6</td>
<td>100.0 ± 10.7</td>
<td>100.0 ± 17.0</td>
<td>100.0 ± 13.6</td>
</tr>
<tr>
<td>ATRPi-SAM</td>
<td>13.1 ± 3.3</td>
<td>6.4 ± 2.3</td>
<td>34.4 ± 5.8</td>
<td>8.7 ± 1.5</td>
<td>6.4 ± 3.6</td>
</tr>
<tr>
<td>OEG-ATRP</td>
<td>9.8 ± 3.0</td>
<td>0.6 ± 0.1</td>
<td>2.1 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>CB/OEG-ATRP</td>
<td>7.0 ± 3.2</td>
<td>1.2 ± 0.2</td>
<td>12.6 ± 1.2</td>
<td>3.0 ± 0.4</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td>PC/OEG-ATRP</td>
<td>6.9 ± 4.6</td>
<td>0.7 ± 0.3</td>
<td>9.4 ± 1.8</td>
<td>3.5 ± 1.0</td>
<td>1.8 ± 1.9</td>
</tr>
<tr>
<td>PC-ATRP</td>
<td>17.2 ± 6.2</td>
<td>1.7 ± 0.7</td>
<td>11.0 ± 2.9</td>
<td>3.1 ± 0.9</td>
<td>6.3 ± 2.8</td>
</tr>
<tr>
<td>CM5</td>
<td>1.2 ± 0.5</td>
<td>0.2 ± 0.1</td>
<td>2.8 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>0.4 ± 0.3</td>
</tr>
</tbody>
</table>

Table 4-17. Stably adsorbed model proteins listed in Table 4-11 on surfaces listed. Listed values are expressed as percentages of adsorbed protein observed upon the M-SAM surface. Error has been calculated as the 95% confidence interval where n = 12.
Figure 4-17. Graphical comparison of protein adsorption upon three dimensional interfaces listed in Table 4-14; ATRP-SAM, CMS, OEG-ATRP, CB/OEG-ATRP, PC/OEG-ATRP, PC-ATRP. All surfaces were tested in the presence of p20 surfactant at 0.005% mass volume.

Of the three-dimensional surfaces the commercial CMS chip was most resistant to irreversible protein fouling. Relative to this surface all ATRP surfaces bound a greater mass of protein. None of the ATRP polymer coatings increased the extent of protein fouling relative to the ATRPi-SAM. Where average adsorption values are qualitatively higher than the ATRPi-SAM (i.e. lysozyme on PC-ATRP and BSA on CB/OEG-ATRP) the differences are not significant (unpaired t test p=0.263 and p=0.557).

Adsorption across the ATRP surfaces appears to follow a general trend where adsorbed mass decreases with increasing protein molecular mass. This contrasts with the predicted correlation of adsorption on two-dimensional surfaces (Figure 4-12). To test this relationship the data is re-presented in Figure 4-18 using a log-log scatter plot.
Figure 4-18. Non-selective adsorption of model proteins upon three-dimensional ATRP surfaces. For each point $n = 12$ over three separate surfaces, with 95% confidence intervals indicated. Proteins plotted increase in mass (lowest to highest): lysozyme, bovine serum albumin; IgG and fibrinogen. The plotted formula attempts to correlate the percentage of a monolayer adsorbed with protein molecular mass using a power function. There is a correlation between the extent of fouling and the molecular mass of the protein upon these three-dimensional surfaces. Values omitted from the presented fit are displayed as open, uncoloured circles. The justification in each case is provided in the accompanying text.
In contrast to the SAM surfaces, adsorption across ATRP surfaces is inversely correlated with protein molecular mass. This correlation is strongest with OEG-ATRP and PC-ATRP surfaces while only single point deviations are evident in the trends for CB/OEG-ATRP and PC/OEG-ATRP. When these points are discounted the remaining points are strongly correlated. These data points have been omitted from the trend lines plotted in Figure 4-18 though the points themselves have been plotted and described in the figure text.

4.5 Investigating the Effect of Molecular Species within the Buffer upon Protein Adsorption at the Sensing Interfaces

4.5.1 Testing the Effect of Surfactant p20 on the Fouling of the M-SAM Model Interface

Following the aggregation issues experienced with lysozyme and IgG on the M-SAM, when surfactant was necessarily omitted (section 4.3.2), the surfactant p20 was added to all subsequent adsorption tests (throughout section 4.3.3). While pragmatic to prevent protein aggregation, this introduced another variable into the comparative testing of different surface chemistries as the surfactant may interact with the surfaces to an unknown extent and through potentially different mechanisms. Especially considering the methacrylate backbone of the ATRP polymers will be more solvent exposed relative to the alkyl chain of the SAM surfaces. It may, therefore, be pertinent to investigate the effect of surfactant p20 on surface performance.

In order to understand the potential change in system behaviour the non-specific adsorption screening of the M-SAM was repeated with surfactant p20 added at the same concentration used in the general testing (0.005% mass.volume⁻¹). The relative extent of non-selective adsorption is presented in Table 4-18. It is clear the surfactant changes the behaviour of the interface drastically as protein adsorption upon the same M-SAM surface is reduced by two orders of magnitude.
<table>
<thead>
<tr>
<th>Surface</th>
<th>Lysozyme (% monolayer)</th>
<th>Fibrinogen (% monolayer)</th>
<th>Sheep Serum (% monolayer)</th>
<th>Rabbit IgG (% monolayer)</th>
<th>BSA (% monolayer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-SAM - p20</td>
<td>100 ± 18.9</td>
<td>100 ± 6.6</td>
<td>100 ± 10.7</td>
<td>100 ± 17.0</td>
<td>100 ± 13.6</td>
</tr>
<tr>
<td>M-SAM + p20</td>
<td>1.5 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>4.5 ± 1.0</td>
<td>0.7 ± 0.1</td>
<td>3.0 ± 0.6</td>
</tr>
</tbody>
</table>

Table 4-18. Comparison of fouling from the non-selective binding panel as a percentage monolayer upon a methyl terminated SAM surface in the presence or absence of surfactant p20. Error has been calculated as the 95% confidence interval where n=12.

On a hydrophobic surface such as the M-SAM the p20 surfactant is reported to self-assemble forming a dense layer. It may be anticipated that this layer be readily displaced by proteins which would themselves adsorb to the interface: this is clearly not the case, at least for the concentrations of proteins used here. This level of resistance warrants further investigation. Re-presenting the data as a log-log scatter plot (Figure 4-19) may indicate whether the surfactant coating is behaving as a planar two-dimensional interface akin to the SAM surfaces, or as a three-dimensional interface akin to the ATRP polymer brushes.
A negative correlation is found between protein molecular mass and the extent of adsorption on the M-SAM+p20 interface. This is surprising and it suggests a size exclusion effect is occurring due to the p20 coating over the hydrophobic M-SAM interface. It is possible the other planar surfaces interact with p20 and so the same re-presentation of SIA and ATRPi-SAM data is rendered in Figure 4-20. These surfaces also show a negative correlation which suggests that these surfaces also adsorb surfactant from the solvent. Such an assertion would require verification through repeat testing of these surfaces without p20. This has not been undertaken.

Figure 4-19. Non-selective adsorption of model proteins upon the surfactant coated M-SAM surface. For each point $n = 12$ over three separate surfaces, with 95% confidence intervals indicated. Proteins plotted increase in mass (lowest to highest); lysozyme, bovine serum albumin; IgG and fibrinogen. The plotted formula attempts to correlate the percentage of a monolayer adsorbed with protein molecular mass using a power function. There is a correlation between the extent of fouling and the molecular mass of the protein upon this surface.
The p20 coated M-SAM surface is the most protein resistant interface tested thus far. It may be anticipated that surfactants will be displaced from the surface at some point to be replaced with proteins. Such molecular displacement as must be occurring with the bare SIA interfaces which are likely to interact with the p20 surfactant through the ether oxygens. Incoming protein is clearly capable of displacing this surfactant coating at relatively low protein concentration. A new experiment was devised in order to identify the protein concentration at which surfactant mediated fouling resistance is may be titrated.

In order to titrate the protection level afforded by p20 a dilution series of sheep serum was chosen for two reasons; it was the most recalcitrant sample of those tested in section 4.3, and the numerous proteins of which it is composed are stable at high concentration and so, when surfactant is not present, precipitation which may block the fluidics is less likely to occur. However, the use of serum precludes any detailed mechanistic analysis, though it does represent a most challenging sample type and will allow convenient investigation of effects as they relate to protein concentration and heterogeneity. Figure 4-21 therefore presents two titration experiments presented as overlain SPR sensograms; M-SAM sheep serum titration with and without p20.
Figure 4-21. Sequential exposure of the M-SAM surface to increasingly concentrated sheep serum (up to 100% sheep serum when $t = 0$ and each exposure preceding this is a three-fold dilution). Titrations take place with or without p20 surfactant (red trace = with p20 surfactant at 0.005 % v/v, grey trace = without any surfactant). Fouling from the sheep serum is quickly observed when surfactant is omitted but when present p20 surfactant resists fouling effectively.

The overlain sensograms (Figure 4-21) present serial surface exposures each with a successive three-fold increase in serum concentration from $\sim 150$ ng.ml$^{-1}$ up to $\sim 80$ mg.ml$^{-1}$ (undiluted sheep serum). The first injection is buffer and the second starts the series of serum injections. Where present the p20 surfactant is at a constant concentration in all samples, including whole sera (mass.volume$^{-1} = 0.005\%$). In order to compare adsorption resistance between the two surfaces it is pragmatic to compare stably absorbed protein which may be plotted as a bar chart (Figure 4-22).
It is evident from Figure 4-22 that in the absence of the surfactant p20 adsorption starts to occur at a low protein concentration and begins to plateaus at a relatively moderate concentration (~1% serum concentration). In contrast when p20 surfactant is present adsorption is retarded, though the protection provided by adsorbed p20 layer begins to be eroded at moderate serum concentration (~3% serum).

4.5.2 Testing the Effect of Surfactant p20 on the Fouling of Three-Dimensional Interfaces

Three candidate three-dimensional interfaces were tested using the sheep serum titration method. The titration results are presented in Figure 4-23 where p20 surfactant was added to, or omitted from, the running buffer.
Figure 4-23. Sequential exposure of the M-SAM surface to increasingly concentrated sheep serum (up to 100% sheep serum). Titrations take place with or without p20 surfactant (solid coloured bars = with p20 surfactant at 0.005 % v/v, hatched bars = without any surfactant). Significant adsorption is indicated (determined by paired t-test result of the indicated injection in relation to the immediately preceding injection). n = 4 with replicates coming from within the same surface, 95% confidence intervals indicated.
There are two interesting points in each serum titration within Figure 4-23; the titrating serum concentration (i.e. the concentration where fouling becomes detectable), and the extent of fouling upon exposure to whole serum. It is evident that p20 surfactant does not affect the ATRP interfaces to the same extent as it does the M-SAM. When surfactant is omitted adsorption is detectable at a lower serum concentration upon both CM5 and OEG-ATRP, though only at a 9-fold and 3-fold concentration reduction respectively. No difference was observed in the initial fouling concentration of PC-ATRP. The extent of serum adsorption, however, is markedly affected and all surfaces adsorb a greater mass of serum components when surfactant is omitted. This suggests that the extent of fouling measured in section 4.3 may be expected to be influenced by the presence of p20 surfactant though the broad trends of interaction identified are not likely to change (i.e. charge based interfaces still foul to the greatest extent).

4.5.3 Testing the Effect of Buffer Type on Fouling upon the PC-ATRP Interface

It is possible that ionic additives have an effect on the ionic interfaces. Hepes buffer has been used throughout and in order to briefly test the idea sheep serum titrations were repeated upon the PC-ATRP using phosphate buffered saline, without p20 surfactant (Figure 4-24). The different buffers generated no detectable effect on the extent of adsorption; there was no significant difference between the extent of adsorption from serum diluted into either buffer (unpaired t tests p>0.05 for all injections).
**Figure 4-24.** Sequential exposure of the PC-ATRP surface to increasingly concentrated sheep serum (up to 100% sheep serum). Titrations take place without p20 surfactant and serum being diluted into two different buffers, and these buffers being used as running buffers, also (■ phosphate buffered saline, ■ hepes buffered saline). Significant adsorption is indicated (determined by a paired t-test result of the indicated injection in relation to the immediately preceding injection). n = 4 with replicates coming from within the same surface, 95% confidence intervals indicated.

### 4.6 Discussion

**Surface design, fabrication and characterisation:** The successful fabrication of all interfaces is testament to the level of control afforded by alkanethiol self-assembly. This enabled the fabrication of two-dimensional surfaces and subsequent surface initiated polymerisation for the fabrication of three-dimensional surfaces. Following fabrication deposited coatings were characterised using goniometry, XPS, ellipsometry and AFM. All surfaces were broadly as designed with the exception of the OEG-ATRP interface which was thinner than anticipated. This irregularity did not reduce the value of this interface as a reference surface. Comparison of literature references to the OEG-SAM [131] and CB/OEG-SAM [133] interfaces prepared herein suggests either the surface density of these SAMs or perhaps their topography is in variance with these prior reports. The fact these surfaces were piranha cleaned immediately prior to use may have damaged the surfaces [139] and increased the heterogeneity. An alternative cleaning method could have been used which would be interesting to compare (e.g. sulfochromic surface preparations [139]). The cause and consequence of this variance is unknown.
A compromise in the fabrication of these interfaces was the chemistry incorporated through which protein recognition elements were coupled. The use of a common coupling method across all interfaces was desired though technical limitations precluded this (e.g. SAM stability and methacrylate availability). The pragmatic solution was to use different conjugation methods for the SAM and ATRP surfaces; to SAM surfaces carboxyl groups were added to allow conventional EDC/NHS conjugation [98], and to ATRP surfaces biotin groups were added to allow streptavidin conjugation [129]. These inter-surface differences are not thought to have substantively changed surface fouling behaviour, though exceptions have been observed.

A further compromise was accepted in the characterisation of the ATRP surfaces where a potentially critical interface parameter was untested: polymer chain density of the ATRP surfaces. Many studies infer chain density as exact measurement is not convenient. For example, sacrificial initiators included in the solution phase (used to propagate the surface polymer) may be easily characterised via mass spectrometry. Such approaches are inaccurate as length is overestimated and polydispersity underestimated [140] with the result that density is underestimated and heterogeneity ignored. Practical methods have been recently reported for silica surfaces which cleave the grafted chains from a solid silica substrate allowing quantitative analysis of the surface grafted chains [141]. While informative, specific conclusions from any substrate/method are potentially limited to the method/substrate used (as they may relate to the density of surface confined initiators upon that substrate, e.g. silica). As such new methods would be required for the gold substrates used here. Herein the lack of quantitative polymer density characterisation may confound comparison of polymer performance and mechanistic determination.

However, empirical comparison of refractive index does allow comparison between the ATRP surfaces tested here, refractive index being reportedly correlated with chain density. It is not possible to calculate chain density from this data even though dry film thicknesses are known as the extent of their swelling behaviour upon hydration is not. As a result any calculations combining the two would be unreliable. The relative refractive index across the ATRP series does allow inference of chain densities even though quantitation is precluded. This inference of relative chain densities is drawn upon in the discussion concerning the classification of protein fouling upon the ATRP coatings.

**The random sequential adsorption model:** The adsorptive behaviour of the model reference system – the methyl terminated M-SAM – is described by a random sequential adsorption process [136]. This model system proved an appropriate reference for all surfaces tested. The extent of adsorption upon all surfaces could therefore be normalised by reference to this data as percentage monolayers; as undertaken by Chapman et al. [134].
Proteins as physico-chemical probes: The model proteins used to test interface fouling resistance were selected as molecular probes being of different charge/size. Those selected are well reported for this purpose. The proteins did serve as generic physical probes though some variations were observed where apparently specific interactions occurred. For example, the capacity of IgG to interact non-specifically with charged species resulted in elevated levels of adsorption on any surface displaying freely accessible charged functionalities (e.g. the OEG-SAM). In addition BSA exhibited variable adsorptive behaviour on hydrophilic and hydrophobic interfaces, suggesting it may interact non-specifically through different mechanisms (which may be anticipated considering its native role as a sorptive courier of heterogeneous solutes in vivo).

While specific chemical-functional behaviours were observed it is clear that, from the broad adsorption trends observed across two- and three-dimensional interfaces, the physical behaviour of the proteins dominates their surface interaction. The advantage of using several protein probes, as evidenced herein, is that variance due to any specific chemical process (e.g. IgG charge interaction) may be identified and separated from any overriding physical processes and physical trends may be readily identified (e.g. size-exclusion). The greater number of probes used the better and no fewer than four would be recommended.

The impact of p20 surfactant on adsorption upon designed surfaces: The addition of p20 surfactant to the experiment was pragmatic in order to avoid spontaneous protein aggregation, though the consequence was potentially uncontrolled. However, p20 was found not to interact appreciably with the OEG-SAM or CB/OEG-SAM. When equilibrated in surfactant containing buffer the refractive indices of these surfaces were proportional to the mass of the anticipated SAM, suggesting that no substantive interaction between surfactant and SAM was occurring.

p20 interaction was found to significantly change the binding propensity of serum to the non-ionic OEG-ATRP surface but not for the ionic PC-ATRP surface. It is possible that this surfactant may have disproportionately affected the adsorption behaviour of the non-ionic ATRP surface. The implication being that fouling resistance of this surface may have been suppressed in the adsorption screening. This is not thought to appreciably change the implications of the data and trends discussed further below.

The fouling behaviour of the M-SAM was modified profoundly by the presence of surfactant p20. This behaviour is discussed later in this section.

Protein fouling at two-dimensional SAM interfaces: Relative to non-ionic functionalities the ionic components within SAMs have the effect of increasing non-selective protein adsorption. This was the case for all ionic functionalities within the SAM surfaces, whether a major or minor component of the surface.
Ionic functionalities provide a stronger force of interaction [142] and so their use may be anticipated to require exacting fabrication methods to avoid inhomogeneities or defects, e.g. localised charge clusters. Holmlin et al. reported surfaces related to the CB/OEG-SAM, being composed of only the ionic components, and found them to be more resistant to protein exposures of identical concentration [133]. The surfaces herein contained OEG tethers for antibody immobilisation and so they are different to those reported in the Holmlin study. The reduced resistance observed here may be attributable to surface imperfections introduced by the inserted tethers or by differences within fabrication methods and storage conditions. However, as the carboxyl of the OEG tether was found to be a fouling entity for IgG it is most likely that fouling is explicitly due to the ionic species present.

Across each SAM type protein adsorption was observed to occur at a broadly consistent ratio across the size range of proteins tested (excluding IgG), i.e. the C1 carboxylate surface formed ~1.2% of a monolayer regardless of protein molecular mass, OEG-SAM ~1.9% and CB/OEG-SAM ~7.6%. This is in agreement with trends reported by Chapman et al. where carboxylate SAMs demonstrated fractional adsorption of 1% across a range of protein molecular masses [134]. The apparent uniformity of fractional adsorption suggests there may be a predetermined limit/propensity for these surfaces to bind/coordinate protein.

The consistent formation of diffuse fractional monolayers upon the hydrophilic SAM surfaces requires that something is operating to limit surface densities upon these surfaces. The M-SAM reference system is anticipated to be uniformly adsorptive, i.e. the probability that a protein may adsorb is anticipated to be equally likely across the two-dimensional surface. The resulting adsorption density is thought to be self-limiting due to progressive steric occlusion from adsorbing species. Assuming an even distribution of molecules adsorb upon the other SAMs tested these adsorbed proteins cannot be physically occluding each other as they are too diffuse.

Forces compete and counteract in any system. In the case of protein adsorption on the M-SAM surface the dehydration of the hydrophobic interface is an overriding force driving adsorption. When the hydrophobic methyl termination is replaced with hydrophilic functional groups (e.g. the OEG-SAM or CB/OEG-SAM) the balance of adsorptive interactions necessarily changes as a result of more favourable intermolecular interactions with the aqueous solvent. Without the dominating hydrophobic force it is possible that comparatively weaker forces may become a more evident upon surface-protein interactions.

It is possible that the mechanism of adsorption upon hydrophilic surfaces is actually related to that of hydrophobic surfaces; it may be mediated indirectly by the solvent. This mechanism would require that solvating water upon the hydrophilic interface be geometrically/chemically incongruous with bulk water. The free energy of non-polar hydrophobic hydration can be negative, and therefore favourable. However,
the hydrating molecules orient in an irregular manner relative to the structure of water within the bulk volume and so this favourable energy is counteracted by bulk water. Hydrogen bonds are directional and the extent of the geometric discrepancy between hydrating water and bulk water stresses the hydrogen bonding network which propagates across the interface creating transient hot-spots of chemical potential. Hydrophobic non-polar functionalities have a most profound impact whereas hydrophilic polar functionalities engage the aqueous solvent more efficiently. The continuum of water engagement between these two extremes is attributable to the ability of specific functional groups to coordinate hydrating molecules. The capacity of coordinated molecules to geometrically engage with the bulk solvent through hydrogen bonds may drive interfacial fouling behaviour at hydrophobic surfaces and it is probable that other more subtle hydrogen bonding phenomena are important in hydrophilic adsorption.

Considering hydration free energy it is possible that, although the hydrophilic functionalities have a favourable enthalpy of hydration, their extensive and artificial two-dimensional repetition may create a geometrically imposed entropic penalty. Interfacial hydrogen bond dynamics within the first hydration layers may be experimentally interrogated using sum frequency infra-red spectroscopy. Studies of aqueous hydrophilic and hydrophobic interfaces suggest hydrating water molecules have insignificantly different hydrogen bond lifetimes. This suggests that the entropy of directly hydrating layers is independent of surface chemistry and that subsequent and successive hydration layers must therefore determine the chemical potential of the supramolecularly engaged solvent around the interface. This being the case, due to the cooperative nature of hydrogen bonds, suboptimal hydrogen bonding may propagate through successive hydration layers. The different fractional adsorption densities of two-dimensional surfaces may then be explained by the varying chemical potential of the solvating water upon the different functional groups employed.

It is possible that proteins which adsorb to the hydrophilic SAM’s become extensions of the planar surface, three-dimensional non-covalent surface adducts condensed upon the surface to ameliorate the chemical potential of the solvent. Upon adsorption the chemical potential of surface engaged water is neutralised and further protein adsorption therefore has no explicit driver. The spontaneous exchange of adsorbed proteins may therefore be anticipated. This mechanism may therefore explain the Vroman effect, where mixtures of proteins of different sizes appear to dynamically and spontaneously reorganise themselves upon a solid-liquid interface [143].

The mechanism underpinning the Vroman effect remains undefined and may be explained by aqueous chemical potential. For example, consider a mixed solution of two proteins of different size at the same molar concentration, upon surface exposure to the mixture the smaller of the two proteins will diffuse to the surface with exponentially greater speed and so the initial coating will predominantly be composed of.
the smaller protein. The Vroman effect describes the process by which the smaller protein is progressively replaced by the larger protein. If the chemical potential of the solvent is mediating protein adsorption then such exchange may be spontaneous, with the initial protein coating and matured coating being thermodynamically equivalent. In such systems hydrogen bonded water networks propagate from the surface and encompass all adsorbed proteins. As such water molecules are vibrationally coupled into a contiguous, dynamic three-dimensional web which is cohesive and elastic. These networks inadvertently pin proteins to the surface as they minimise interfacial chemical potential by using the proteins to displace focal points of sub-optimal hydrogen bonding. Pinned proteins may be spontaneously displaced by other proteins which passively diffuse to the interface. A protein diffusing toward the surface may increase the water activity of locally solvating molecules. As the vibrational dynamics surrounding adsorbed and incoming proteins become equivalent this may allow one protein to spontaneously decouple from the surface and simply diffuse away from it. Once the displaced protein migrates from the interface the substituting larger protein becomes pinned to the surface as water activity reduces once more. Through such a spontaneous process it is a statistical probability that larger proteins may be more effective at substituting smaller proteins as the effective affinity of their surface interaction is greater. In balancing the energy of the system the density of the larger proteins may be reduced relative to the smaller proteins, as observed here.

The concept of “interphase” water and its consequence for interfacial protein behaviour was proposed by Vogler in 1998 [97]. This has been criticised for lacking practical evidence to support it [144] but the fouling upon the carboxyl SAM observed in this work (and that of Chapman et al. where additional proteins were applied [134]) can have no other explanation. The density of proteins adsorbing to the carboxyl interface is reduced by two orders of magnitude and at such ranges the proteins are unlikely to have any direct lateral interaction. Mediation by the solvent interaction with the surface seems most likely. The quality of surface solvation seems to be of consequence. Studies of other systems have indicated similar. A bilayer membrane was formulated with varying concentrations of cholesterol and tested for human serum albumin adsorption [145]. Protein adsorption upon the zwitterionic lipid interface was suppressed at 10-30% cholesterol composition but was observed when cholesterol was at higher or lower concentrations, the implication being the surface density of ionic species changes and thus the solvent behaviour changes, too. This indicates that the nature of the surface density of hydrophilic functionalities is important and this likely relates to the quality of hydration which may result.

This may be practically investigated by comparing the extent of fractional monolayers formed in the presence of salts from the Hofmeister series. Sodium chloride, used herein, may simply be substituted for alternative salts purported to increase or decrease the relative order of hydrogen bonding networks. It
may be anticipated that salts increasing hydrogen bonding order (e.g. sodium citrate) may reduce the extent of surface fouling.

Protein fouling at three-dimensional ATRP interfaces: SPR measures mass adsorbed within the local three-dimensional environment of the two-dimensional gold interface (i.e. the first 150-200 nm of the gold surface). It provides no inherent capacity to discriminate between primary, secondary or ternary adsorption (as defined by [146]). However, deductive reasoning across the series of chips tested here, and in the context of literature reports, may allow inference of fouling locations across these surfaces.

If ternary adsorption were to have occurred then, relative to the underlying ATRPi-SAM interface, it would likely manifest as a substantive increase in adsorbed mass per unit area across the interface. This effect has been demonstrated in the development of ion exchange media prepared via ATRP, and subsequently validated monitoring adsorption of protein (BSA) in real-time using SPR [147]. Substantive adsorption was not observed on any of the ATRP surfaces tested here, while it may be occurring it is not at any appreciable extent.

Primary adsorption may be occurring upon the ATRPi-SAM, i.e. upon the underlying surface between grafted polymer chains. The extent of lysozyme adsorption across all ATRP surfaces is not significantly different from the ATRPi-SAM. It is therefore possible that lysozyme is uniformly penetrating all ATRP polymer brushes. The evident size exclusion effect, where the extent of fouling by larger proteins is reduced, may be explained by a size distribution and frequency of pores across the polymer coating resulting from the random ATRP initiation/propagation deposition process.

An alternative explanation is that the terminations of the propagating chains are responsible for the adsorption of lysozyme. This would mean adsorption upon the ATRP brushes is secondary. As observed with two-dimensional surfaces, consistent fractions of protein monolayers can form upon chemically uniform interfaces (proposed to result as a function of solvent chemical potential) and it is possible that the plane of propagating methacrylate chains may present such an interface. This would explain the similarity in lysozyme adsorption across all surfaces. The observed size-exclusion effect may then be explained as a function of surface topography. Though the chemical potential of hydrating solvent is equivalent across the terminating functionalities the frequency of physical binding loci across the surface, where topography is sufficiently complimentary to accommodate proteins, may be expected to reduce as protein size increases.

The critical difference between primary and secondary adsorption mechanisms is in the density of chains on the ATRP surfaces. Primary may be predominant upon diffuse brushes and secondary upon dense brushes. The refractive index of the CB/OEG-ATRP surface indicates that chain density upon this surface is
significantly higher than the other ATRP surfaces (potentially 50% higher than the other ATRP surfaces). The fouling occurring upon this interface is more extensive; both lysozyme and BSA adsorb to the same extent upon this interface as they do upon the ATRPi-SAM. This indicates that fouling is most likely of secondary nature as if it were primary the resistance of this interface may be anticipated to be reduced as a result of the significantly higher chain density. The author therefore believes that fouling of ATRP is secondary. Verification of the fouling location is required and would be possible by neutron reflection studies of deuterated proteins (as undertaken with PEG brushes [148]).

Many ATRP graft polymers have a correlation between fouling resistance and chain length [138]. In context of the observations above, this length effect may be explained by one of two prospective mechanisms. Primary adsorption may be reduced as any gaps in surface coverage may be more effectively screened by gyrating solvated chains. Alternatively secondary adsorption may reduce as brush polydispersity increases generating greater irregularity in surface topography and allowing intra-chain rearrangement to minimise interfacial free energy. It has been demonstrated that brush polydispersity does increase with length [149] and it is most likely that the reported increase of fouling resistance with polymer length, is a function of this. Polydispersity is thought to affect the surface energy of the brushes, as indicated by goniometry: water contact angle changes were found to correlate with fouling resistance [138]. Inoue and Ishihara attributed this correlation to the greater mobility of the diffuse chains in the boundary layer between bulk solvent and dense brush, which may self-organise to minimise interfacial free energy by shielding the hydrophobic chain terminations. Short polymer brushes (akin to the 5 nm films used here) were thought to be strongly influenced by the chemistry of the chain termination. The effect was dependent upon the monomers incorporated; pHEMA showing little effect while a carboxybetaine polymer was strongly affected by length. This supports the assertion that fouling upon short ATRP brush surfaces relates to the termini of the closely packed chains and is therefore likely to be secondary in nature.

Longer, reportedly resistant, ATRP polymer coatings tend to be in the order of 25 nm in the dry state. When swollen with aqueous buffer such polymer coatings typically double their volume. The consequence for SPR sensors is that the most sensitive evanescent zone upon the gold surface is populated with a dense brush which excludes analytes. So while fouling resistance is increased sensitivity is reduced. While thick coatings will inevitably reduce SPR assay sensitivity there is a more significant limitation: fouling propensity of low molecular mass species is likely to increase.

While long polymer brush interfaces may screen secondary adsorption from large proteins they may have the inadvertent effect of increasing the propensity of secondary/ternary fouling from smaller proteins. This has been observed (though not explained) in size exclusion chromatography where small proteins
were found to be retarded by solid phase media to an extent that could not be explained by size-exclusion alone [150]. A possible explanation is that the chains within the coating are dense and polydisperse, as the protein diffuses into the brush interface it interacts through additive non-specific three-dimensional surface contacts within the nano-confined hydrated brush environment. Polydispersity may therefore inadvertently create many non-selective yet high affinity binding loci for lower molecular mass materials.

This adsorption is likely a consequence of the low mobility water engaged by the polymer [151]. In effect the low mobility aqueous brush becomes a discrete phase into which other species may partition and in-so-doing increase the water activity of the system by displacing coordinated, nano-confined water. Such phase separation is driven by the chemical potential of the engaged solvent. This phenomenon has been observed for biological materials across length scales and in different energetic systems; from simple peptides partitioning into immobilised nano-scale OEG brushes to quaternary proteins partitioning into extensive aqueous two-phase systems. Chemical potential is likely to manifest upon all aqueous interfaces, however, by minimising the depth of the solvated plane it may be possible to minimise both the propensity and extent of such adsorptive processes as the volume of engaged water and cooperativity of interactions may be reduced.

**Chemical vs physical fouling resistance mechanisms:** It seems peculiar that fouling resistant interfaces remain elusive while many models have predicted that three-dimensional polymer chains would do the job should they be suitably physically defined (i.e. immobilised in high density) and chemically defined (i.e. composed of hydrophilic functionalities) [93, 152, 153]. Theoretical physical concepts have matured but they have yet to translate into the greatly anticipated practical solutions. So what is the limitation in their realisation? Such models fixate upon physical properties and do not account for the chemical behaviour of water, specifically its anisotropic and cooperative hydrogen bonding. While this omission allows the expedient development of simple, and undeniably informative, physical models the convenient simplification disregards the chemical properties of aqueous systems which fundamentally dominate all adsorptive processes occurring within them, like non-covalent biological adhesion.

The theoretical superiority of chemical (i.e. two-dimensional) or physical (i.e. three-dimensional) interfacial protein adsorption resistance has been debated at length in the literature. It is clear from the data herein that these are interdependent and practically inseparable. The Whitesides group used SAMs to dissect the role of chemical identity in protein adsorptive behaviour upon two-dimensional interfaces. However, the co-dependence of polymer chemical functionality and physical mechanics cannot be sensibly abstracted for comparable systematic interrogation. The identification of ideal functionalities, and their requisite chain densities, therefore remains an empirical experimental challenge as aqueous systems defy predictive computational modelling (again, owing to complexity of anisotropic cooperativity in hydrogen
bonding). The convoluted experimental challenge will therefore endure to simultaneously interrogate the interdependent properties of hydrophilic functionality, molecular structure, surface density, polymer length, etc.

The empirical search for new materials assumes that in order to realise theoretical models we require as yet unknown components to be identified. This is improbable. These models ignore molecular hydration and will likely never translate into resistant structures, at least where water is the solvent. It is more likely that alternative fabrication methods are required to apply existing components in a new way. OEG was the most fouling resistant SAM surface prepared by the Whitesides group and is one of the most well studied biocompatible materials. The propensity of OEG to coordinate water is well known, as is its capacity to integrate into the solvent structure as a hydrated helix, and it can accommodate a hydrophilic terminus, or any other terminus. Categorisation of the OEG-SAM herein as a two-dimensional interface is accurate when scaled against proteins, but when scaled against a water molecule it would be more accurately described as a three-dimensional interface. The concomitant chemical and physical behaviour of this polymer suggests it is well suited to fouling resistant applications. The chemical interaction with water is essential but so too is its helical conformation which acts as a physical scaffold for successively engaged hydration layers. Neither chemical nor physical properties have primacy it seems. All surfaces presenting OEG were found to be more resistant relative to their charge based counterparts, in either two- or three-dimensional architectures. These OEG interfaces fouled to different extents and this suggests that the manner in which OEG is presented is of fundamental significance. This is dictated by the fabrication method. OEG may well possess the requisite functionality, it may simply be a matter of how it is applied to the interface which needs to be further investigated.

**Antibody immobilisation within the interfaces:** The aim of immobilising antibodies within the SPR interface is to incorporate functional binding sites in high density. Relative to adsorbed antibody upon the gold interface both immobilisation strategies applied here (i.e. direct coupling via amines and indirect via amine biotinylation) generally achieved higher densities of antibodies with low variability between immobilisations. The only exception to this was the CB/OEG-SAM upon which it was not possible to control immobilisation. This is not surprising considering the propensity of this interface to bind antibody non-selectively which will have subverted immobilisation control.

Comparison of binding activities between the two chemical immobilisation strategies indicated a clear difference; the indirect biotinylation immobilisation process reduced the activity of the antibody by half relative to direct amine coupling. Another source of binding inactivation appears to be the length of the PEG tether employed. Although PEG tethers were employed across all custom fabricated interfaces, while short OEG tethers (e.g. E=6 employed in the B-SAM interfaces) accommodated antibody in a dense but
accessible manner, the OEG-ATRP tethers (where E=15) were found to further reduce binding activity. This suggests that the tethers themselves may be interfering with the binding function of the antibody. This effect may relate to the solvated structure of the polymer sterically occluding antibody binding sites or it may result from explicit interaction of the tether with the antibody. This may equally be explained by direct antibody interaction with the surface though this seems unlikely considering the general resistance of this interface to non-selective antibody interaction. Such aberrant interactions may, however, be cooperative and once the antibody is confined to the surface interactions with tether(s) or methacrylate chains may change. Whatever the cause the apparent inhibition of antibody binding appears attributable to either the length of the PEG tether or the proximity of the methacrylate backbones upon the ATRP surfaces.

It was generally observed that when antibody interacts non-specifically with the underlying surface control over protein behaviour is reduced leading to irregular immobilisation (e.g. upon CB/OEG-SAM) and/or compromised binding activity. It is therefore a general prerequisite that surfaces must not exhibit any explicit interaction with the antibody in order to control its behaviour. This rules out any charge based interfaces tested here.

Where antibody was immobilised upon such an interface, and not sterically confined or inhibited by PEG, high binding activities were observed (e.g. OEG-SAM). The accommodation of the antibody in a three-dimensional hydrogel (e.g. the CMS chip) allowed more extensive saturation of the evanescent sensing filed, however, such interfaces were found to foul in complex or concentrated sample types. The implication being that, in order to reduce non-selective interaction, the underlying interface may need to present a contiguous interface of hydrated functionalities (akin to the OEG-SAM) which may only permit the accommodation of a monolayer of antibodies. Reported attempts to employ diffuse pOEGMA hydrogel structures suffered from non-specific fouling. It is possible that in this reported case the underlying OEG-SAM interface failed, as observed in section 4.2.3, though it is also possible that fouling may be occurring to the central methacrylate chains/pendant functionalities as size-exclusion has been designed-out of this interface. It seems unlikely that diffuse hydrogel structures will be capable of resisting non-selective fouling on account of the general phase separation phenomenon which may override confined local excluded-volume effects.

Charge based interfaces exploiting carboxybetaine have been reported and successfully applied within SPR imparting the joint functionalities of a zwitterionic non-adsorbing interface with integral carboxylic acid groups for facile protein immobilisation. These surfaces do adsorb material however as within the reported methods employing them they are blanked with serum or other proteins. This may be appropriate when the nature of the fouling species is known (e.g. clinical samples such as serum) but
where they are unknown (e.g. environmental samples) such an approach may be less reliable for environmental applications resistance must be complete.

Should a two-dimensional interface be engineered for resistance, and accommodate protein recognition elements, there is no guarantee that either the interface or the antibody will continue to resist non-specific interaction. Fouling resistance of the antibody coated interfaces was not tested. The extent of antibody mediated non-specific adsorption may be minimised by the use of recombinant techniques to reduce the size of the immobilised antibody, as described in section 3. However, the very fact that the recognition element is a protein may increase the extent of fouling from materials present in the sample matrix, and this may be independent of the underlying interface. Hydrophobic components within the sample (e.g. soil colloids) are likely to adsorb to the antibody in the same way as the protein panel were observed to foul the M-SAM interface in the previous section. This represents another challenge in the development of techniques for the analysis of unknown samples without the encumbrance of sample processing: proteins incorporated within the surfaces may foul too.

Adaptive interfaces – a forgotten concept for fouling resistant interfaces: Of all the fouling resistant interfaces tested the most interesting is the M-SAM surface when coated with p20 surfactant. This interface was the most resistant of all interfaces fabricated. This non-covalent and self-assembled coating reduced the extent of protein adsorption from the model protein panel by two orders of magnitude, reducing it to ~1% of a monolayer. In addition, relative to the uncoated M-SAM, the surfactant coating exhibited a modest size-exclusion effect, only observed upon three-dimensional ATRP surfaces.

It is possible that the non-covalent surfactant coating is capable of efficiently reducing the chemical potential of the solvent at the solid-liquid phase boundary in a manner beyond that of the other interfaces. The adaptability of the other interfaces is constrained by the fact they are covalently immobilised, though as discussed above it has been suggested that long ATRP polymers (i.e. >20 nm) are able to reorganise their structure to reduce the solvent exposure of hydrophobic chain termini [138]. The p20 layer is inherently more adaptive and evidently more successful at minimising the free energy of the interface as protein adsorption is not as favourable.

The resistance afforded by the surfactant coating relates specifically to the M-SAM as when other planar surfaces were tested (i.e. SIA and ATRPi-SAM surfaces) fouling resistance was substantively higher (i.e. fractional monolayers ~20% and ~10% respectively). It is therefore possible that the surface energy of the M-SAM interface is important in efficiently resisting fouling by specifically interacting with the p20 surfactant, i.e. through hydrophobic/ dispersion forces. While the resistance afforded by adsorbed surfactant upon the other planar surfaces was not as powerful it is interesting to note that a size exclusion
effect was still apparent. This feature of adsorbed surfactant behaviour therefore appears unaffected by the nature of specific surface interactions. This indicates that the density of surfactant upon these surfaces may equate with the chemical potential of the interface. The efficiency of fouling resistance may therefore be a function of surfactant affinity for the surface and the propensity of the surfactant to be displaced from the surface by adsorbing protein.

Despite the general application of surfactants in biological detection and diagnostic applications (e.g. ELISA) the concept of engineering non-covalent, adaptive surface coatings to resist fouling in real-time affinity sensor applications is novel. In addition to surface fouling resistance such a concept would allow the general mitigation of fouling from hydrophobic sample contaminants (e.g. soil and soot). Adaptive non-covalent interfaces may provide concomitant solutions for the inhibition of both surface fouling and antibody fouling and this is the subject of further investigation within this thesis.
5. Development of a Surfactant Based Affinity Biosensor Interface

5.1 Aim of this Chapter

The work herein aims to develop a novel affinity biosensor interface based upon a non-covalent self-assembled surfactant layer to resist all non-selective biological interaction (i.e. protein adsorption), into which functional biological recognition elements (i.e. antibodies) may be integrated.

A hybrid covalent/non-covalent interface is developed. A novel non-covalently, self-organising oligoethylene glycol surfactant layer is assessed for resistance to all detectable non-selective adsorption from undiluted animal serum. Within this dynamic coating polyethylene ether tethers are covalently immobilised carrying pendant biotin molecules as a putative recognition element.

5.2 Interface Design Based on Non-Covalent Surfactant Self-Assembly

The development of covalent surface modification techniques (exemplified in Chapter 4) follow the assumption that covalent control is required. Such control is useful, to systematically test interfacial parameters in research and development, but this has become a generally accepted rule. No such approach has delivered a resistance interface. Evidently, there remain significant gaps in our understanding about what properties are fundamentally required of interfaces to control the physicochemical behaviour of biological materials in such environments.

The biomaterials literature largely discards non-covalently coated interfaces [154] considering them to be unsuitable as biologically compatible interfaces but this assumption has not been critically examined. This may be the case for some applications (e.g. implanted biomaterials) but not all. Observations have been reported where surfactant coatings afford surprising levels of biological inertness [155] but these tend not to be further interrogated.

A potentially profound and largely oblique assumption within covalent deposition is that solvents are interchangeable, i.e. solvents used in the assembly of interfacial architectures have no role to play in their behaviour after synthesis, that they may be switched out as required for other solvent/solute systems. As nonpolar solvents are fundamentally irrelevant to the molecular systems the biointerface will be operating in, why use them as thermodynamic scaffolds through which to assemble such a critical physicochemical structure? Practically, it is convenient to do so in order to control the chemistry within deposition. This makes it a technologically pragmatic approach, but not necessarily a sensible scientific one. While covalent approaches have been the principle route to validate theoretical models and explore the enabling
chemistry such approaches are attempting to craft an intricate three-dimensional scaffold to integrate an alien material seamlessly into the aqueous energetics of biological systems. To try and build such an interface in the absence of water is rather hopeful.

Chapter 4 found the most resistant interface was in fact a non-covalent, self-assembled coating of surfactant p20 when assembled upon a hydrophobic methyl terminate SAM surface. This demonstrated the intriguing possibility that it may indeed be possible to use the chemical potential of an aqueous phase boundary to self-assemble surface coatings that may subsequently minimise the chemical potential of the interfacial system, creating a more resistant and uniform interface relative to covalent strategies. Adsorbing surfactant molecules may themselves tessellate as the initially adsorbed monolayer matures according to the free energy of the aqueous interfacial system. Surfactant organisation is therefore driven by the same processes driving the surface adsorption of biological materials (e.g. solvent cohesion). It would be an elegant solution to resolve non-specific protein adsorption by co-opting the very force which drives it to assemble a thermodynamically preferential non-covalent assembly.

In order to co-opt such non-covalent surfactant assemblies for affinity biosensor interfaces biological recognition elements must be functional when integrated into the coating. This may be undertaken via two strategies indicated in Figure 5-1; non-selective adsorption of an antibody to the underlying interface (akin to ELISA), or conjugation of the BRE to a molecular tether integrated into the interface. OEG would be a suitable polymer tether for integration into a non-covalent surfactant coating for two reasons; surfaces based on OEG were the most resistant of those tested in Chapter 4, and it is itself intrinsically resistant to biological adsorption. Either interface design may be simply fabricated via hydrophobic SAM deposition followed by surfactant equilibration and BRE immobilisation (or vice versa for BRE adsorption to the interface).
5.3 Comparison of Non-Selective Serum Adsorption Resistance Afforded by Different Surfactants Self-Assembled upon an Alkyl Surface

5.3.1 Characterisation of Surfactant p20/Rabbit Serum as a Reference System

Whole serum was used herein to titrate surfactant resistance and determine the extent of fouling from whole serum. The serum titration method demonstrated in Chapter 4 used whole sheep serum. However, further use in this work was precluded as the stock material experienced freeze-thaw cycling and its protein concentration reduced as material flocculated/precipitated. Further use was therefore avoided. In order to standardise the serum adsorption titration method, to allow comparison of different surfactants, freshly prepared and commercially available rabbit serum was used in place of the sheep serum. The prime advantage of the new method being that this fresh serum was processed once (thawed and filtered at 0.45 µm to remove aggregates) and frozen in single-use aliquots (in method 2.2.11). Comparison titrations between the new rabbit serum and the original sheep serum used in Chapter 4 is presented in Figure 5-2.
Figure 5-2. Sequential exposure of Biacore HPA surface to increasingly concentrated rabbit serum (up to 100% serum). Titrations take place with or without p20 surfactant at a concentration of 0.05 % mass.volume. n = 4 with replicates within the same surface, 95% confidence intervals indicated. Absorption from the sheep serum appears to occur with greater propensity and surface density. As observed in Figure 4-22, p20 surfactant does reduce fouling profoundly but the resistance of the non-covalent film is titrated eventually.

As may be anticipated from the work in Chapter 4 the effect of p20 is profound when added to either serum preparation; the surfactant suppresses protein adsorption to the hydrophobic interface. There is a clear difference between the adsorptive behaviour of sheep and rabbit serum samples. Fouling from the sheep serum is greater than the rabbit serum. The sheep serum is the more challenging sample type though this may be attributable to artefacts of laboratory handling (i.e. the additional freeze-thaw cycling). The relevance of this difference is unknown, though the freeze thaw cycling of biological materials is thought to denature them and this may be anticipated to make surface fouling behaviour more pronounced.
5.3.2 Comparative Resistance Afforded by Alternative Surfactants

Cationic or anionic surfactants were not tested on account of their inherent propensity to bind proteins cooperatively and physically denature them. As the physical structure of the analyte is probed by biological recognition elements and this character must be preserved as denaturation is likely to subvert this. So, by design, surfactants within this putative affinity biosensor interface must not denature the physical character or behaviour of any biological materials, especially the immobilised antibodies!

Three representative zwitterionic surfactants were compared (Figure 5-3) of two general surfactant structures (i.e. single and double aliphatic chain structures) and two zwitterionic functional groups (i.e. phosphorylcholine and carboxybetaine). While these surfactants did reduce the extent of fouling compared to surfactant-free serum, they all demonstrated inferior adsorption resistance relative to p20. All zwitterionic surfactant coated surfaces fouled to a greater extent and were titrated at a lower serum concentration. This suggests that when surfactants with zwitterionic functionalities are adsorbed upon a hydrophobic interface they are less effective at resisting fouling relative to the non-ionic surfactant p20.
Figure 5-3. Sequential exposure of a surfactant/lipid equilibrated (at a concentration of 0.05 % mass-volume) Biacore HPA surface to increasingly concentrated rabbit serum (up to 100% serum). Molecular species as illustrated with border colour to correlate to the bar chart. n = 4 with replicates within the same surface, 95% confidence intervals indicated. Propensity of absorption from the rabbit serum increases with concentration. Single chained surfactants are more resistant to protein adsorption than double chained surfactant.

All non-ionic surfactants tested reduced the extent of serum fouling relative to surfactant free serum adsorption (Figure 5-4). Relative to p20 the only surfactants affording greater resistance were Triton X-100 and Pluronic F-127. Interestingly Brij-35, chemically related to these more resistant surfactants, is not itself resistant which suggests there are structural requirements for surfactants to impart adsorption resistance. Fouling of Brij-35 does not appear to be clearly attributable to either hydrophile or hydrophobe. The hydrophile is shorter than that within Pluronic F-127 and the hydrophobe is identical to that found within p20. Polyethylene glycol surfactants appear to be uniquely resistant to fouling though it is clear that the structure of these surfactants is important and merits further investigation.
Figure 5-4. Sequential exposure of a surfactant equilibrated (at a concentration of 0.05 % mass-volume) Biacore HPA surface to increasingly concentrated rabbit serum (up to 100% serum). Comparative rabbit serum titration of non-ionic surfactants. Surfactant species named in the key as a range of non-ionic surfactant (various structures employing polyethylene glycol/oligoeylene glycol or saccharides). n = 4 with replicates within the same surface, 95% confidence intervals indicated. Propensity of absorption from the rabbit serum increases with concentration. Oligoethylene glycol surfactants are the most resistive of serum fouling.

5.4 Investigation of Polyethylene Glycol Surfactant Features/Properties Required for Adsorption Resistance

5.4.1 Length of Polyethylene Glycol Hydrophile

Having determined that PEG containing surfactants reduced the extent of fouling most appreciably the effect of PEG length was investigated. Triton X-100 is one of a series of surfactants with varying PEG length and so the longer Triton X-350 was compared to Triton X-100 (Figure 5-5) in rabbit serum titration. While Triton X-100 is largely resistant to serum adsorption the longer PEG within Triton X-305 is not as resistant.
Figure 5-5. Sequential exposure of a surfactant equilibrated (at a concentration of 0.05 % mass-volume) Biacore HPA surface to increasingly concentrated rabbit serum (up to 100% serum). Comparative serum titration of Triton non-ionic surfactant series (general structure inset with key). n = 4 with replicates within the same surface, 95% confidence intervals indicated. Propensity of absorption from the rabbit serum increases with concentration. Surfactants with long PEG appear to foul to a greater extent.

Brij-35 (C₁₂E₂₃) is part of a homologous series of CₓEᵧ surfactants and so to confirm the apparent PEG length effect a shorter homologue, C₁₂E₆, was compared. Relative serum adsorption is presented in Figure 5-6. Two features are evident; the serum concentration where fouling is first observed, and the maximal fouling observed in undiluted serum. Intriguingly C₁₂E₂₃ is more resistant at lower serum concentrations (i.e. below 11% serum) where C₁₂E₆ is more resistant at high serum concentrations (i.e. above 11% serum). Adsorption occurring in the presence of C₁₂E₆ appears to plateau at ~50 pg:mm² whereas, under the same experimental conditions, C₁₂E₂₃ does not reach saturation.
Figure 5-6. Sequential exposure of a surfactant equilibrated (at a concentration of 0.05 % mass.volume⁻¹) Biacore HPA surface to increasingly concentrated rabbit serum (up to 100% serum). Comparative rabbit serum titration of non-ionic C\(_{12}\)E\(_{6}\) surfactants: C\(_{12}\)E\(_{6}\) and C\(_{12}\)E\(_{23}\) (also known as Brij-35). n = 4 with replicates within the same surface, 95% confidence intervals indicated. Propensity of absorption from the rabbit serum increases with concentration. Surfactants with long PEG appear to foul to a greater extent but the shorter chained surfactant adsorbs protein too, but whatever binding can occur is rapidly saturated at the level indicated by the dashed grey line.

The length of PEG clearly has an impact upon surfactant behaviour; the longer the PEG the greater the propensity for serum components to adsorb to the interface. The surface density of longer PEG surfactants may be more diffuse as the physical size and shape of the solvated PEG chain may preclude close packing (in a good solvent PEG will form a random coil with a radius of gyration described by the Flory radius). The observed fouling may therefore result from serum components penetrating a more diffuse layer of longer PEG surfactant. It may be possible to test this if surfactant layers presenting long PEG chains are mixed with a shorter PEG surfactant that may increase the chain density of the surfactant layer and therefore restore resistance to the coating. To this end a series of concentration ratios of C\(_{12}\)E\(_{6}\) and C\(_{12}\)E\(_{23}\) were compared through rabbit serum titration (Figure 5-7).
Sequential exposure of a surfactant equilibrated (at a concentration of 0.05 % mass./volume) Biacore HPA surface to increasingly concentrated rabbit serum (up to 100% serum). Comparative rabbit serum titration of non-ionic C_{12}E_{x} surfactants and mixtures thereof: C_{12}E_{6} and C_{12}E_{23} (also known as Brij-35). n = 4 with replicates within the same surface, 95% confidence intervals indicated. Propensity of adsorption from the rabbit serum increases with concentration. Mixed surfactants foul in proportion to their concentration.

The mixed formulations appear to moderate the features identified in Figure 5-6; the presence of C_{12}E_{23} increases fouling resistance at lower serum concentrations (i.e. below 11% serum), whereas the presence of C_{12}E_{6} reduces the extent of fouling at high serum concentrations (i.e. above 11% serum). Formulations of the two surfactants partially combine the benefits of each but the fouling which is observed appears proportional to the relative concentration of each surfactant in any specific formulation (Figure 5-8). There is therefore no apparent cooperativity between the two surfactants in mediating fouling resistance and the fouling which is occurring in the presence of each surfactant is explicitly attributable to it. Each surfactant may indeed by binding different components from the serum, or it may be the local environment of the surface/surfactant which is binding non-selectively.
Figure 5-8. Re-presented data from Figure 5-7 indicating that the extent of serum fouling correlates with the concentration of each surfactant present in the mixture. A) Extent of rabbit serum fouling when 9-fold diluted which is proportionate to and therefore attributable to C_{12}E_6 within the mixture. B) Extent of rabbit serum fouling when undiluted (i.e. 100%) which is proportionate to and therefore attributable to C_{12}E_{23} within the binary mixture. This indicates that the mixtures do not have any greater intrinsic resistance to fouling relative to simple single component formulations, i.e. no synergistic relationship is observed.

Pluronic F-127 presents a PEG chain length of \(~100\) ethylene repeats and is more resistant to serum fouling. This suggests that the PEG chain is unlikely to be adsorbing serum components directly and that the fouling of the C_{12}E_{23} coated surface occurs at or in close proximity to the surface/hydrophobe and that even when mixed with the shorter C_{12}E_6 surfactant this fouling still occurs. The hydrophobic functionality of the surfactant, and its associative behaviour with the surface and homologous surfactant molecules, is
therefore potentially implicated. Taken together these observations indicate that the PEG chain is essential but its relation to the hydrophobe is important.

5.4.2 Chemistry and Structure of the Hydrophobe

Examples of the \( \text{C}_x\text{E}_y \) surfactant series were used to probe the effect of hydrophobe length in surfactant serum titration (Figure 5-9). The PEG chain was of consistent length (\( n = 8 \)) while the carbon number of the alkyl chain increased. With an alkyl chain length of 8 carbons the behaviour of the surfactant is highly variable which may be attributable to the phase behaviour or aggregate structures of this surfactant on a planar interface. The addition of two carbons to the alkyl hydrophobe reduces this variability. As the carbon chain length increases from 10 to 14 fouling appears to reduce.

Figure 5-9. Sequential exposure of a surfactant equilibrated (at a concentration of 0.05 % mass.volume\(^{-1}\)) Biacore HPA surface to increasingly concentrated rabbit serum (up to 100% serum). Comparative serum titration of \( \text{C}_x\text{E}_y \) non-ionic surfactant series (general structure inset), specifically tested homologues were; \( \text{C}_8\text{E}_5 \), \( \text{C}_{10}\text{E}_5 \), \( \text{C}_{12}\text{E}_5 \) and \( \text{C}_{14}\text{E}_5 \) (bar chart coloured according to the inset key). \( n = 4 \) with replicates within the same surface, 95% confidence intervals indicated. Propensity of adsorption from the rabbit serum increases with concentration. Surfactants with longer alkyls such as \( \text{C}_{14}\text{E}_5 \) appear to foul to a lesser extent while shorter alkyl chains such as \( \text{C}_8\text{E}_5 \) are more prone to fouling and the response is inherently more variable.
The size of the hydrophobe appears important in the assembly of a resistant coating of PEG containing surfactant. In order to further investigate the role of the hydrophobe alternative chemistries were tested which have found utility in applications outside of conventional biological assays. PEG surfactants exploiting silane and perfluoroalkane hydrophobic functionalities are used as crop spraying and paint additives respectively. Serum titrations of commercial formulations of L-77 and Zonyl FSO-100 are presented in Figure 5-10.

![Figure 5-10](image_url)

*Figure 5-10. Comparative rabbit serum titration of surfactants. Comparative rabbit serum titration of non-ionic surfactants Silwet L-77 and Zonyl FSO-100 in comparison with p20. Sequential exposure of an equilibrated Biacore HPA surface to increasingly concentrated rabbit serum (up to 100% serum). Titrations take place with surfactant at a concentration of 0.05 % mass.volume⁻¹. n = 4 with replicates within the same surface, 95% confidence intervals indicated. With p20 surfactant, propensity of adsorption from the rabbit serum increases with concentration. With Silwet L-77 whatever binding occurs is rapidly saturated at a level of around 10 pg.mm⁻². Zonyl FSO-100 indicates practically no fouling from 100% serum.*

Silwet L-77 and Zonyl FSO-100 are more resistant to serum adsorption than the C₆E₄ series or p20. Interestingly they are similar to Triton X-100.
5.5 Investigating the Relationship Between Surface Hydrophobic Chemistry and the Hydrophobic Functionality of the Surfactant

5.5.1 Production and Characterisation of Surfaces Supporting Self-Assembled Monolayers of Hydrogenated or Perfluorinated Alkanethiols

As observed in section 4.4.1 the underlying surface energy of the solid interface makes a difference to the serum resistance of non-covalently self-assembled surfactants. A strong interaction between p20 and surface was promoted by the use of a hydrophobic SAM surface whereas gold was unable to maintain the p20 coating when challenged with serum. To further investigate the role of surface energy serum titration may be undertaken on surfaces of different chemistries using surfactants of different hydrophobic chemistry.

In order to assemble interfaces presenting PEG tethers (as envisaged in Figure 5-1) is may be desirable to deposit them using the solvent which they will ultimately operate within in any subsequent assay. This means that alternative methods are required for SAM production using water in place of ethanol. The use of water to deposit a hydrophobic coating is counterintuitive though deposition in water where a surfactant has been used to solubilise the hydrophobic material has been reported. A method was therefore developed to use water and surfactant blend to deposit hydrophobic SAMs on gold with the view that this method would allow the integration of PEG tethers which would be well hydrated in the end application (method 2.3.1).

Following the aqueous emulsion method 2.3.1, hydrocarbon and perfluorocarbon SAMs were fabricated. These are 1-Undecanethiol and 1H,1H,2H,2H-Perfluorodecanethiol, denoted C11H and C10F respectively. A reference surface was also included; gold without any thiol though exposed to the same conditions (e.g. aqueous surfactant blend). Goniometry was undertaken to characterise the resulting surfaces. Figure 5-11 shows water contact angles upon different surfaces and indicates that the surfaces have different surface energies. Comprehensive water contact angle data is presented in Figure 5-12 and shows there is a significant difference between the different surface treatments and that reproducibility within each surface treatment is high.
Figure 5-11. Water contact angle analysis for gold SIA surfaces prior to, and following aqueous surface deposition (via method 2.3.1) of 1-Undecanethiol and 1H,1H,2H,2H-Perfluorodecanethiol. n = 3 for each water angle measurement upon each of three surfaces tested for each surface treatment. Each surface repeat is separately presented in addition to the mean (n = 9). 95% confidence intervals indicated.

Goniometry of these surfaces with alternative solvents (Figure 5-12) allows the calculation of surface energies. For gold, hydrocarbon and perfluorocarbon these were 59.2 mN.m⁻¹, 30.7 mN.m⁻¹ and 18.5 mN.m⁻¹ respectively (values calculated by Dr Corrinne Stone, DSTL).
While the deposition of these SAMs does create reproducible and significantly different surface energies it is necessary to compare their performance to those which were fabricated using a conventional solvent (e.g. ethanol). For direct comparison of chip performance the adsorption of the model protein panel from section 4.3 was repeated (Table 5-1). A similar level of performance is observed in non-selective binding of p20 surfactant non-covalently coating the two M-SAM surfaces indicated in Table 5-1. When compared with ethanol deposited surfaces the significance of any difference is as follows; unpaired t-tests of adsorbed lysozyme p=0.158, fibrinogen p=0.288, dilute sheep serum p=0.024, IgG p=0.07 and BSA p=0.822. The only significant difference in protein adsorption between the two surfaces is sheep serum. Unexpectedly, it was the surface deposited using an aqueous surfactant formulation which was more resistant to serum adsorption.
<table>
<thead>
<tr>
<th>Surface</th>
<th>Lysozyme (%) monolayer</th>
<th>Fibrinogen (%) monolayer</th>
<th>Sheep Serum (%) monolayer</th>
<th>Rabbit IgG (%) monolayer</th>
<th>BSA (%) monolayer</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-SAM (ethanolic)</td>
<td>1.5 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>4.5 ± 1.0</td>
<td>0.7 ± 0.1</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>M-SAM (aqueous)</td>
<td>1.0 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>2.2 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>3.1 ± 0.4</td>
</tr>
</tbody>
</table>

Table 5-1. Comparison of fouling resistance of M-SAM coated with p20 when the hydrocarbon SAM is fabricated in an aqueous emulsion or in a conventional ethanol solvent. The method is taken from Chapter 4, section 4.6.2. Fouling from the non-selective binding panel is expressed as a percentage of fouling which occurs on the M-SAM in the absence of surfactant p20. Error has been calculated as the 95% confidence interval where n = 12 spread across three different surfaces.

This functional comparison of aqueous/ethanolic deposition method demonstrates that they appear comparable though this comparison does not characterise the structure of the SAM created (i.e. fine structure of the two-dimensional crystal) which may be anticipated to be different. It is, however, apparent that aqueous surfactant assisted deposition can yield surfaces with a comparable capacity to recruit surfactant from the buffer and resist protein adsorption and that these surfaces may therefore be used to compare the capacity of different surfactants to coat and protect surfaces with different surface energies.

### 5.5.2 Relative Non-Selective Binding Resistance Afforded by Surfactants of Different Hydrophobic Functionality

The surfaces developed in section 5.5.1 were tested for their propensity to adsorb rabbit serum. As a reference system no surfactant was added. This data is presented in Figure 5-13. The level of adsorption between the two hydrophobic SAMs is comparable and replicates the same mass of adsorption observed from sheep serum upon the M-SAM in section 5.5.2. The difference in surface energy between these two hydrophobic surfaces does not affect the adsorption of serum components. 1% serum was not applied to these interfaces which was an experimental omission. The fact that serum adsorption is saturated at the 10% serum is noteworthy as this passivates the interface against further adsorption from the subsequent exposure of 100% serum. This is not the case for the gold interface which adsorbs a greater mass of protein with additional exposure.
Figure 5-13. Bar chart presenting the extent of rabbit serum adsorption upon surfaces prepared following method 2.3.1 over a series of sequential exposures at the concentrations indicated. Three different surfaces (see inset key) were exposed in a Biacore 2000 at a flow rate of 20 ul.min⁻¹ for 10 minutes. The extent of adsorption was recorded 60 seconds after the exposure had finished and the surface had re-equilibrated in running buffer. The final exposure was to SDS detergent in order to determine how reversible the serum fouling was. n=12 spread across three separate surfaces with four points being tested on each, 95% confidence intervals are indicated. In this experiment 1% serum exposure was omitted in error though this was included in subsequent experiments. Surface fouling of the hydrophobic SAMs at 10% serum v/v forms a saturated coating and no further fouling is observed at 100% rabbit serum. SDS detergent cleans adsorbed serum components from the hydrophobic SAMs but not the gold.

When surfactant p20 is added to the system serum adsorption is greatly reduced (Figure 5-14). As the concentration of the challenge increases the level of detected adsorption also increases. This is most notable for the gold surface which suggests the surfactant has an affinity for gold though it is more easily displaced from this surface than it is either of the hydrophobic surfaces. Of the hydrophobic surfaces the p20 resists fouling to a greater extent when adsorbed onto the hydrocarbon surface.
Figure 5-14. Bar chart presenting the extent of rabbit serum adsorption derived using SPR (Biacore 2000). Hydrophobic surfaces (prepared following method 2.3.1) were equilibrated in hepes buffered saline containing surfactant p20 (at a concentration of 0.05% mass.volume⁻¹) followed by a series of sequential exposures at the concentrations indicated. Three different surfaces (see inset key) were exposed in a Biacore 2000 at a flow rate of 20 ul.min⁻¹ for 10 minutes. The extent of adsorption was recorded 60 seconds after the exposure had finished and the surface had re-equilibrated in running buffer. The final exposure was to SDS detergent in order to determine how reversible the serum fouling was. n=12 spread across three separate surfaces with four points being tested on each, 95% confidence intervals are indicated. The presence of p20 surfactant suppressed rabbit serum fouling upon all surfaces though it was most effective upon hydrophobic SAMs. Adsorption did however occur but upon the hydrophobic SAMs this could be partially removed by SDS detergent.

The data presented in Figure 5-14 is reference data to compare the alternative surfactants. The comparative fouling behaviour of Silwet L-77 is presented in Figure 5-15.
Figure 5-15. Bar chart presenting the extent of rabbit serum adsorption derived using SPR (Biacore 2000). Hydrophobic surfaces (prepared following method 2.3.1) were equilibrated in hepes buffered saline containing surfactant Silwet L-77 (at a concentration of 0.05% mass-volume\(^{-1}\)) followed by a series of sequential exposures at the concentrations indicated. Three different surfaces (see inset key) were exposed in a Biacore 2000 at a flow rate of 20 ul.min\(^{-1}\) for 10 minutes. The extent of adsorption was recorded 60 seconds after the exposure had finished and the surface had re-equilibrated in running buffer. The final exposure was to SDS detergent in order to determine how reversible the serum fouling was. n=12 spread across three separate surfaces with four points being tested on each, 95% confidence intervals are indicated. The presence of L-77 surfactant suppressed rabbit serum fouling upon all surfaces though it was most effective upon hydrophobic SAMs. Adsorption did however occur but upon the hydrophobic alkane surface this could be removed entirely by SDS detergent.

Similar to p20 the siloxane surfactant L-77 reduces the extent of adsorption, though adsorption suppression on the gold interface is not apparently different to that achieved with p20. The level of resistance achieved when the underlying interface is hydrophobic is greater. A difference between the hydrocarbon and perfluorocarbon surfaces is also apparent with the hydrocarbon resisting fouling to a greater extent. Cleaning of adsorbed serum from alkyl surface was complete.

Relative to the p20 surfactant the resistance afforded by the surfactant Zonyl FSO-100 (Figure 5-16) appears less effective on the gold surface but more effective on the hydrophobic interfaces. The two hydrophobic interfaces appear mutually resistant.
Figure 5-16. Bar chart presenting the extent of rabbit serum adsorption derived using SPR (Biacore 2000). Hydrophobic surfaces (prepared following method 2.3.1) were equilibrated in hepes buffered saline containing surfactant Zonyl FSO-100 (at a concentration of 0.05% mass.volume⁻¹) followed by a series of sequential exposures at the concentrations indicated. Three different surfaces (see inset key) were exposed in a Biacore 2000 at a flow rate of 20 ul.min⁻¹ for 10 minutes. The extent of adsorption was recorded 60 seconds after the exposure had finished and the surface had re-equilibrated in running buffer. The final exposure was to SDS detergent in order to determine how reversible the serum fouling was. n=12 spread across three separate surfaces with four points being tested on each, 95% confidence intervals are indicated. The presence of FSO-100 surfactant suppressed rabbit serum fouling upon all surfaces though it was most effective upon hydrophobic SAMs. Adsorption did however and SDS detergent was not effective in removing this.

There is a clear requirement for the underlying interface to be hydrophobic to enable surfactants to coat the surface and then resist fouling. There is also some benefit in having favourable interactions between the hydrophobic surface and the surfactant (probably mediated by synergistic electron densities and the ensuing VdW interactions), but this is secondary to the role of the surfactant hydrophobe in the formal efficiency of the surfactant.
5.6 Insertion of a Biological Recognition Element into the Surfactant Interface by Sequential Adsorption onto the Underlying Hydrophobic Surface

The simplest method to incorporate a biological recognition element within the surfactant interface is to adsorb it to the underlying hydrophobic surface prior to equilibration with surfactant (see Figure 5-1-A). This method is widely used when preparing immunosorbent 96 well plates for ELISA. While it was possible to very simply immobilise antibody upon the hydrophobic surface, when Zonyl FSO-100 surfactant was introduced the adsorbed protein was gradually eluted from it (Figure 5-17).

![Figure 5-17](image.png)

**Figure 5-17.** Bar chart of SPR sensor response of the practical implementation of schematic 5-1-A: adsorbed antibody upon a hydrophobic surface surrounded by non-covalently coordinated surfactant. The sensing interface is a Biacore HPA chip coated with murine monoclonal antibody (as per method 2.2.8) which binds *Bacillus anthracis* spores and has been equilibrated in hepes buffered saline containing Zonyl FSO-100 surfactant at a concentration of 0.05% mass-volume. A series of proteins spiked into either running buffer or whole rabbit serum is presented (see inset key). The presented response is attributable to each individual exposure (i.e., the values are independent of each other having been baselined separately). No regeneration of the interface was attempted between each exposure as this may strip the surface of antibody. Significant variance is observed in SPR responses. Antibody is actively eluted from the interface by the surfactant as indicated by the consistently negative SPR responses. Non-specific adsorption from rabbit serum is observed but it is modest. n = 4 with all points sampled from the same interface, 95% confidence intervals are indicated.
Despite the elution of the antibody detection of a target protein within whole serum was attempted as it is possible the binding response would be of sufficient magnitude to be detected above the rate of loss from the unstable antibody coated surface. This would demonstrate that the antibody retained function within the surfactant film. The adsorbed antibody was raised against ricin toxin and the detection of toxin spiked into whole serum was attempted. Non-specific adsorption from the serum, and elution of the antibody from the surface, is evident in Figure 5-18 but the antibody was shown to bind ricin and detect it at a concentration of 1 µg.ml⁻¹.

Figure 5-18. Bar chart of SPR sensor response of a practical implementation of schematic 5-1-A: adsorbed antibody upon a hydrophobic surface surrounded by non-covalently coordinated surfactant. The sensing interface is a Biacore HPA chip coated with murine monoclonal antibody (as per method 2.2.8) which binds Ricin toxin and has been equilibrated in hepes buffered saline containing Zonyl FSO-100 surfactant at a concentration of 0.05% mass.volume⁻¹. Significant variance is observed in SPR responses due to antibody desorption from the surface. Antibody is eluted from the interface by the surfactant as indicated by the consistently negative SPR responses. Relative to the BSA controls within this test, and the antibody control surface presented in Figure 5-17, Ricin toxin is detected at a concentration of 1 ug.ml⁻¹. This is denoted with the symbol *. n = 4 with all points sampled from the same interface, 95% confidence intervals are indicated.
The instability of the adsorbed antibody interface precludes its further use. The losses from the surface would be continuous in an on-line affinity biosensor and the variance created by such losses would degrade sensor performance (i.e. leading to false negatives). So an alternative method is required for the integration of biological recognition elements. However, it is noteworthy that monoclonal antibodies are functional in the presence of the Zonyl FSO-100 surfactant.

5.7 Integration of a Biological Recognition Element into the Surfactant Interface via Covalent Polyethylene Ether Tether

5.7.1 Insertion of a Heterofunctional Biotin-Lipoamide Polyethylene Ether Tether into the Hydrophobic Self-Assembled Monolayers

In order to further develop the SAM interface (demonstrated in section 5.5.1) to integrate a PEG tether a heterofunctional PEG molecule was used bearing a lipoamide at one terminus and a biotin at the other (shown schematically in Figure 5-19). Following method 2.3.2, lipoamide is anticipated to adsorb to the gold spontaneously projecting the biotin terminus into the aqueous surfactant deposition solvent. The surrounding surface may then be backfilled with hydrophobic hydrocarbon/perfluorocarbon. It was hoped that this fabrication method would create a mixed SAM composed of hydrophilic tethers with a hydrophobic SAM understory which, when placed within an aqueous environment, adsorb surfactant which may self-assemble upon it.

![Figure 5-19. Biotin-OEG_{11}-Lipoamide is 53.7 Å in length. When adsorbed upon the gold SIA surface this should be able to penetrate the self-assembled surfactant layer and present the biotin terminus among the surrounding OEG surfactant chains.](image_url)

Initially a gold surface was coated with the tether without further modification to verify the performance of the biotin and understand effects of surface density on biotin/streptavidin interaction. The selectivity of binding upon this interface was tested using streptavidin and BSA; streptavidin should bind the biotin
specifically while the BSA is physically similar to streptavidin in term of size and isoelectric point, and may indicate any issue from non-specific interactions.

The binding responses of a series of interfaces, partnered with surfactant p20, are presented in Figure 5-20. BSA at 10 µg.ml⁻¹ adsorption was resisted by all surfaces with the exception of the gold surface. Streptavidin adsorption at 10 µg.ml⁻¹ was resisted by the methoxy terminated tether interface suggesting that a lipoamide functionalised surface does not foul non-selectively (at this protein concentration). On the biotin tether functionalised surfaces the binding density of streptavidin increased as the concentration of tether in the deposition solution increased (from 750 nM to 7.5 mM). This demonstrates that the tether functions to bind streptavidin selectively. The density of tether achieved at the highest deposition concentration equates to more than a monolayer of streptavidin. This suggests the tether is able to bind a monolayer and then free tether is able to penetrate this layer and coordinate a secondary layer of streptavidin.
Figure 5-20. Specific and nonspecific protein interactions upon an affinity interface composed of: biotin OEG₆₅ tethers with associated aqueous p20 surfactant. Derived using SPR (Biacore 2000). SIA gold surfaces coated (as per method 2.3.2). Prior to protein adsorption surfaces were equilibrated in buffer with surfactant p20 (at a concentration of 0.05% mass.volume⁻¹) and then exposed sequentially to a series of injections; firstly BSA @ 10 µg.ml⁻¹, Streptavidin @ 10 µg.ml⁻¹, whole rabbit serum, and finally SDS detergent to attempt to clean off any surface adsorbed proteins. The plotted responses are attributable to each individual exposure as indicated. Streptavidin binds specifically to the surfaces carrying biotin functionalities. Rabbit serum binds to all surfaces to varying degrees. SDS detergent is successful at stripping adsorbed serum from the surfaces carrying a high density of OEG tether (e.g. when coated with 7.5 mM tether in the deposition process) but less so from those which did not. n = 4 with all points taken from the same surface, 95% confidence intervals are indicated.

The BSA adsorption test was useful to verify that streptavidin binding was selective, however, at only 10 µg.ml⁻¹ it was a very modest test. In order to further test the fouling propensity of these surfaces they were also exposed to whole rabbit serum. It is important to note here that this test was undertaken following exposure to streptavidin and so it is possible that serum fouling may occur to the bound streptavidin in addition to the underlying surface. Serum fouling was most abundant upon the gold surface and upon the other surfaces was found to reduce with greater tether coverage (inferred by the increased density of streptavidin selectively bound). As the extent of serum fouling reduces with greater streptavidin surface density it is a reasonable to assume that serum was not binding appreciably to the streptavidin.
The backfilling of the gold surface with hydrophobic SAMs is required to recruit surfactant from the buffer and induce its self-assembly to form a fouling resistant coating. Tethers pre-adsorbed upon gold surfaces were backfilled with either hydrocarbon (Figure 5-21) or perfluorocarbon (Figure 5-22) based alkanethiols according to method 2.3.1. Surfaces were equilibrated with p20 surfactant prior to any protein interaction testing. Relative to the gold tether series (Figure 5-20) both backfilled SAM series’ contain two additional reference surfaces; the SAM without tether and the tether without the SAM.

Figure 5-21. Specific and nonspecific protein interactions upon a novel interface composed of: biotin OEG₁₁ tethers backfilled with undecanethiol and aqueously, non-covalently coordinated p20 surfactant. Derived using SPR (Biochip 2000). SIA gold surfaces coated as per method 2.3.2. Surfaces were equilibrated in buffer with surfactant p20 (at a concentration of 0.05% mass.volume⁻¹) and then exposed sequentially to a series of injections; firstly BSA @ 10 µg.ml⁻¹, Streptavidin @ 10 µg.ml⁻¹, whole rabbit serum, and finally SDS detergent to attempt to clean off any surface adsorbed proteins. The plotted responses are attributable to each individual exposure as indicated. Streptavidin binds specifically to the surfaces carrying biotin functionalities. Rabbit serum binds to all surfaces to varying degrees. SDS detergent is successful at stripping adsorbed serum from all the surfaces backfilled with undecanethiol (or a high density of tether, e.g. 7.5 mM tether in the deposition process) but less so from the untreated gold surface. n = 4 with all points taken from the same surface, 95% confidence intervals are indicated.
BSA at 10 µg.mL\(^{-1}\) is again found to bind to the gold surface, though all other surfaces resist non-selective fouling at such a low concentration challenge. Streptavidin is again resisted by the methoxy terminated tether and found to bind to all tether bearing interfaces. The densities of streptavidin correlate with the concentration of tether within the deposition solution. The addition of a hydrocarbon SAM to backfill the gold/tether interface appears to reduce the density of tether.

Where the backfilling SAM is a perfluorocarbon the density of tether achieved is potentially not as depleted relative to a tether deposited upon gold alone. The significance of this observation is not known.

Figure 5-22. Specific and nonspecific protein interactions upon a novel interface composed of: biotin OEG\(_{11}\) tethers backfilled with perfluorodecanethiol and aqueously, non-covalently coordinated p20 surfactant. Derived using SPR (Biacore 2000). SIA gold surfaces coated as per method 2.3.2. Surfaces were equilibrated in buffer with surfactant p20 (at a concentration of 0.05% mass.volume\(^{-1}\)) and then exposed sequentially to a series of injections; firstly BSA @ 10 µg.mL\(^{-1}\), Streptavidin @ 10 µg.mL\(^{-1}\), whole rabbit serum, and finally SDS detergent to attempt to clean off any surface adsorbed proteins. The plotted responses are attributable to each individual exposure as indicated. Streptavidin binds specifically to the surfaces carrying biotin functionalities. Rabbit serum binds to all surfaces to varying degrees. SDS detergent is successful at stripping adsorbed serum from all the surfaces backfilled with undecanethiol (or a high density of tether, e.g. 7.5 mM tether in the deposition process) but less so from the untreated gold surface. n = 4 with all points taken from the same surface, 95% confidence intervals are indicated.
though the propensity of the fluorocarbon to elute or compete for surface adsorption with the lipoamide may be reduced relative to the hydrocarbon. This may be functionally useful if thermodynamically self-limiting processes can be developed in the aqueous deposition method.

It is evident that either of the hydrophobic SAMs may be used to backfill the tether interface, they both recruit p20 surfactant which in-turn suppresses serum fouling. Of the two SAMs tested the hydrocarbon has been shown to be the most versatile partnering well with a broader range of surfactants to reduce serum adsorption (section 5.5.2). The compromise may be that it appears to reduce tether density at the interface.

5.7.2 Testing the Relationship Between Biotin-Tether Density and Non-Selective Rabbit Serum Adsorption at the Interface

Serum fouling of the tether interface is attributable to the inserted tether. When the fouling observed in section 5.7.1 is plotted against the density of streptavidin immobilised (indicating tether density) fouling is found to be proportional (Figure 5-23). The gold surface is progressively shielded from fouling with increasing tether density, however, both hydrophobic interfaces are fouled to a greater extent with greater tether density. Considering both hydrophobic interfaces (i.e. hydrocarbon and perfluorocarbon) are mutually resistant when coated with surfactant Zonyl FSO-100 (as indicated in Figure 5-16) it is reasonable to assume that fouling is occurring as a consequence of tether integration. It is not possible to determine the precise location of any fouling, i.e. is serum binding to the surface adjacent to the tether, is the surfactant implicated, or is fouling occurring to the tether itself, in which case is it binding to the lipoamide, the PEG or the biotin?
5.7.3 Testing the Relationship Between Surfactant Type and Rabbit Serum Adsorption of the Biotin-Tether Interface

The interaction of the surfactant with the tether/SAM may be influencing serum fouling. Fouling may be reduced by substituting the surfactant and so those demonstrated to have great potential in Figure 5-10 (Silwet L-77 and Zonyl FSO-100) are thus tested. Figure 5-24 shows the resulting correlation between tether density and fouling propensity, regardless of the surfactant used. This fouling test was slightly different to that in Figure 5-23. Serum fouling upon the tethers was determined before streptavidin had
been immobilised. Following exposure of the surface to serum, SDS detergent was used to strip adsorbed material before streptavidin was subsequently immobilised to determine the tether density. Regardless of surfactant employed, and in the absence of pendant Streptavidin, there is a strong correlation between tether density and fouling propensity. This demonstrates that serum fouling is mediated by the tether. The tether itself is, therefore, either directly or indirectly responsible for serum fouling through some mechanism yet to be determined. It could be directly binding to part of the tether, or it could be indirectly facilitating the penetration of the surfactant coating.

Figure 5-24. Scatter plot showing rabbit serum fouling as it relates to biotin OEG$_{11}$ tether density. Derived using SPR (Biacore 2000). The surfaces tested carry biotin tethers backfilled with undecanethiol. They were partnered with a range of surfactants indicated by the inset key; p20 (grey spots), Zonyl FSO-100 (green spots) and Silwet L-77 (yellow spots). It is important to note that, in contrast to the data presented in Figure 5-23, rabbit serum adsorption was determined before streptavidin binding. This required SDS detergent to be used to strip the serum adsorbed in order to subsequently quantify tether density through Streptavidin affinity interaction. Regardless of the surfactant used to partner with the hydrophobic interface, serum fouling was found to be directly proportionate to tether density. For each point plotted n = 1. The data was collected across 5 different surfaces with four points being derived from each (points from each prepared surface cluster on the scatter plot).
5.7.4 Testing the Relationship Between Biotin-Tether Length and Terminus on the extent of Rabbit Serum Adsorption

To examine the effect of tether length and termination on fouling propensity, two biotin/lipoamide tethers of different length (OEG\textsubscript{11} and OEG\textsubscript{23}) were compared with and without pendant streptavidin. The partnering surfactant was Silwet L-77 and the SAM used to backfill the tethers was a hydrocarbon. Rabbit serum fouling was tested before and after Streptavidin protein had been immobilised. SDS detergent was very effective in completely stripping serum from the tether surfaces before Streptavidin immobilisation (as observed previously in Figure 5-21).

Fouling upon the OEG\textsubscript{11} interface broadly correlates exponentially with density (Figure 5-25). The exponential correlation appears unaffected by the chain terminus (i.e. whether biotin or streptavidin). Once tether density increases beyond a threshold where a monolayer of Streptavidin may be immobilised (indicated as a dashed grey line upon Figure 5-25, at a streptavidin mass density of 3500 pg.mm\textsuperscript{2}), the fouling observed appears to reduce. So, when streptavidin density manifests as a multi-layer it starts to occlude the surface and reduce the extent of serum fouling.

For the longer OEG\textsubscript{23} tether the fouling observed before streptavidin is bound is in broadly similar to that observed for the shorter OEG\textsubscript{11} tether (Figure 5-26). However, when streptavidin is bound to the tether fouling is affected to a greater extent. Fouling upon a streptavidin terminated OEG\textsubscript{23} tether is broadly double that of the biotin terminated tether. This occurs at all densities of tether, including those which support a streptavidin layer in excess of a monolayer (indicated again in Figure 5-26 as a dashed grey line).
Figure 5-25. Scatter plot showing rabbit serum fouling as it relates to biotin OEG\textsubscript{11} tether density (derived using SPR). The interface tested is composed of OEG\textsubscript{11}-biotin tether backfilled with C\textsubscript{11}H alkanethiol filler and partnered with Silwet L-77 surfactant. The mass density anticipated for a monolayer of Streptavidin is indicated by a dashed grey line at 3500 pg.mm\textsuperscript{-2}. Fouling is exponentially correlated with OEG\textsubscript{11} tether density. As indicated, blue circles (and exponential fit) depict biotin terminated tether fouling and red circles (and exponential fit) Streptavidin terminated tether fouling. The streptavidin or biotin termination of the tether makes no detectable difference to fouling extent. Each point represents a single point test. 7 surfaces were tested for each condition prepared with different tether concentrations within the deposition process, four points were tested across each surface.

Figure 5-26. Scatter plot showing rabbit serum fouling as it relates to biotin OEG\textsubscript{23} tether density (derived using SPR). The interface tested is composed of OEG\textsubscript{23}-biotin tether backfilled with C\textsubscript{11}H alkanethiol filler and partnered with Silwet L-77 surfactant. The mass density anticipated for a monolayer of Streptavidin is indicated by a dashed grey line at 3500 pg.mm\textsuperscript{-2}. Fouling is directly proportional to OEG\textsubscript{23} tether density. As indicated, blue circles (and linear fit) depict biotin terminated tether fouling and red circles (and linear fit) Streptavidin terminated tether fouling. Fouling upon the streptavidin terminated OEG\textsubscript{11} tether is near twice that of the biotin terminated tether. Each point represents a single point test. 7 surfaces were tested for each condition prepared with different tether concentrations within the deposition process, four points were tested across each surface.
5.7.5 Demonstration that Rabbit Serum Adsorption Inhibits Streptavidin Binding to the Biotin-Tether

Thus far a self-assembling surfactant coating has been developed to resist fouling from proteins at high concentration and broad heterogeneity (undiluted rabbit serum). The underlying hydrophobic SAM, responsible for the surfactant's self-assembly, has then been modified to incorporate a molecular tether with pendant functionality for the integration of a biological recognition element (via Streptavidin). However, this modified interface was found to foul once more and fouling is attributed to the tether. Short tethers were found to foul to a lesser extent. The consequence of this fouling for detection assays is now explored in a putative binding assay where streptavidin is spiked into undiluted rabbit serum as an analyte.

To demonstrate the sensitivity of the interface a solution of streptavidin (at 10 ng.ml\(^{-1}\)) in buffer (containing Zonyl FSO-100) was injected over the tether interface in a series of exposures increasing in length. The extent of bound streptavidin was found to increase in direct proportion to the duration of the exposure (Figure 5-27). If the interface is resistant to fouling then, in a real-time and continuously monitoring SPR sensor, longer integration times may be permitted.

![Figure 5-27](image)

**Figure 5-27.** Scatter plot presenting SPR derived data attributable to specific binding of streptavidin to the OEG\(_{11}\)-tether interface. The surface used is composed of OEG\(_{11}\) tether with undecanethiol as backfilling SAM and surfactant Zonyl FSO-100 to partner the SAM. Binding responses were generated from a series of surface exposures of streptavidin (10 ng.ml\(^{-1}\)) in hepes buffered saline of increasing duration from 5 seconds up to 160 seconds. Flow rate was 60 ul.min\(^{-1}\). There is a linear correlation between exposure length and mass of streptavidin bound. n = 3 with all points coming from the same surface at three independent locations. 95% confidence limits indicated.
To explore the consequence of surface fouling a series of injections was undertaken where whole serum was spiked with streptavidin at 1 ug.ml⁻¹. A higher concentration of streptavidin was used relative to Figure 5-27 to ensure a binding response for streptavidin was observed above the noise. Figure 5-29 expresses the total mass of material bound/adsorbed to the interface as well as the proportions of this response attributable to streptavidin binding and serum fouling. It is clear that at this concentration streptavidin is binding to the interface but the greatest proportion of binding is attributable to the serum. The extent of streptavidin binding is also seen to plateau after an exposure of 40 seconds. This is thought to be the consequence of serum fouling inhibiting further streptavidin binding, rather than saturation of the interface as a reduction in response is modest and SA is a very high affinity binder. Saturation of the interface with streptavidin would see the surface lose sensitivity more sharply, rather than as a slow reduction observed.

![Figure 5-28](image.png)

**Figure 5-28.** Combined bar chart and scatter plot presenting the SPR derived data attributable to binding of streptavidin (spiked into whole rabbit serum) to the OEG₁₁-tether interface. The surface used is composed of OEG₁₁ tether with undecanethiol as backfilling SAM and surfactant Zonyl FSO-100 to partner the SAM. Binding responses were generated from a series of surface exposures of streptavidin (1000 ng.ml⁻¹) in undiluted serum of increasing duration from 10 seconds up to 320 seconds. Flow rate was 60 ul.min⁻¹. The purple bars present the net binding response of the surface after exposure to the Streptavidin spiked serum. The surface was then cleaned with SDS detergent to remove serum fouling but retain the Streptavidin. Through this SDS process the net binding response could be separated into; specific streptavidin binding (blue trace) and non-specific rabbit serum fouling (red trace). Streptavidin is observed to bind to the interface, but whole rabbit serum is observed to bind to a much greater extent and interfere with streptavidin binding with the result that streptavidin binding reaches a plateau after an exposure of 40 seconds.
5.8 Discussion

Non-covalent, self-organising surface coatings can be entirely resistant to protein fouling: Surprisingly, some of the surface coatings assessed here have proven resistant to fouling from a most heterogeneous and concentrated sample type, undiluted animal serum. Surface fouling from this matrix, upon certain surfactant coatings and certain surfaces, has proven undetectable in the SPR sensor employed (Figure 5-10). This is remarkable and indicates that resistant interfaces are a practical reality, and that they may be made simply through the self-organisation of pre-engineered molecular constituents. These may be further engineered and the whole interface, and its component parts, can be engineered to operate together as a cohesive system including; the surface, recognition element, surfactant, solvent, salts, environmental background matter etc. This soft matter system will benefit from iterative evolution rather than individual isolated component developments.

Adsorption resistant surfactants: All surfactants tested reduced the extent of fouling from undiluted rabbit serum. A general property among the most fouling resistant surfactants is that they are non-ionic oligoethylene glycol surfactants and they are reported to reduce the surface tension of water most appreciably relative to the others on test (Table 5-2).

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Water surface tension</th>
</tr>
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<tbody>
<tr>
<td>Triton X-100</td>
<td>30 mN.m⁻¹</td>
</tr>
<tr>
<td>Zonyl FSO-100</td>
<td>18 mN.m⁻¹</td>
</tr>
<tr>
<td>Silwet L-77</td>
<td>20 mN.m⁻¹</td>
</tr>
</tbody>
</table>

*Table 5-2.* Water surface tension of surfactants demonstrated herein to successfully suppress the adsorption of undiluted rabbit serum to a hydrophobic SAM surface. These values should be contrasted with the surface tension of water 72 mN.m⁻¹. There is clearly a strong thermodynamic driver for interfacial water to partition these solutes upon phase boundaries.

The physical chemistry of the surfactant is important: The size of the PEG/OEG chain is of major consequence. The long chains of Triton X-305 and Brij-35 are resistant to protein fouling at low protein concentration but they are overwhelmed at high concentration. Protein penetration of these long PEG/OEG brushes may be statistically inevitable as they may principally present a kinetic barrier to adsorption. As the density of solvated proteins within the aqueous volume above the surfactant layer...
increases, the probability of physical volumetric overlap increases until it crosses a threshold level where the volumes overlap. The three key parameters here are; the lateral density of the brush, the size of the solvated species and its concentration. The brush must therefore be dense enough to size exclude all potentially interfering species. This may explain why the longer PEG/OEG surfactants were resistive at low concentration and fouling at high concentration.

The physical nature of the hydrophobe also affects brush density, though to a lesser extent. The C\textsubscript{12}E\textsubscript{6} and Zonyl FSO-100 surfactants are analogous; one composed of an alkane hydrophobe the other a perfluoroalkane. The density and structure these surfactants generate upon the surface may be influenced by the cross-sectional area of both the hydrophobe and hydrophile. The density of C\textsubscript{12}E\textsubscript{6} may be anticipated to be greater than FSO-100, owing to the lower cross-sectional area of the alkane [159]. This may explain a peculiar but reproducible feature of serum fouling upon C\textsubscript{12}E\textsubscript{6} where fouling is detectable early in the titration but plateaus quickly at a maximum level of 50 pg.mm\textsuperscript{-2} (Figure 5-6). This does not occur with FSO-100. However, the physical proportions of C\textsubscript{12}E\textsubscript{6} mean it is not able to form a uniform lateral brush and instead forms parallel hemi-cylinders upon the interface [160]. It is possible that the quality of PEG/OEG hydration upon this surface is important and the structure of C\textsubscript{12}E\textsubscript{6} may create a moderately frustrated hydrogen bonding structure in the hydration layer. This was proposed as a possible explanation for the partial but reproducible fouling of carboxyl SAMs where 1% of a protein monolayer binds, regardless of the size of the protein applied (Chapter 3 discussion). This is speculative but the adsorption upon the C\textsubscript{12}E\textsubscript{6} surface is slight (50 µg.m\textsuperscript{-2}) and is either attributable to a small species penetrating the brush, or a slight energetic tension in the solvent structure which is abated by the very modest adsorption level. The fact the FSO-100 brush does not adsorb this same low level, and the fact this brush is likely to be more open and planar in structure [159], support the assertion that this relates to a suboptimal hydration effect. So, the density of the chains is important and there is likely to be an upper and lower bound to this. The upper bound for physical density for a PEG/OEG brush likely sits between that achieved by the hemi-cylindrical alkane and the vertically stacked perfluorinated chains. The lower bound of brush density is not known but it should be sufficiently dense to resist ingress of the smallest interferent species (e.g. peptides). Triton X-100 carries a larger hydrophobic group, as does Silwet L-77, both of which are highly effective at resisting fouling but the brush densities they achieve may be lower are therefore susceptible to fouling from small entities or potentially phase separating materials, too.

PEG/OEG is uniquely successful, and the fact it forms a physical brush is clearly essential, but what of the chemistry? It is possible that linear polyionic materials may be applied in a similar way. However, these may be confounded by two potential issues; enthalpy of hydration, and rotational entropy. The enthalpy of hydration may overpower the necessary role of the hydrophobe, and so the efficiency of the surfactant
may not be sufficient to coat the surface with comparable density. Increasing the size of the hydrophobic moiety (adjusting the hydrophilic-lipophilic balance) to compensate may inadvertently reduce the brush density, or increase the thickness of the coating applied. In addition, the rotational behaviour of ionic polymers may also cause issue as, relative to PEG/OEG, these structures are bulky and strongly coordinated to many water molecules. The anticipated reduction in entropy may increase the risk of fouling, but then the water which is strongly coordinated to the ionic species will unlikely be displaced and so perhaps the two effects may counteract each other. Either extreme may defeat polyionic surfactants but for PEG/OEG surfactants these forces seem to be balanced to enable simple self-assembly of stable functional coatings. As a planar brush it would be interesting to explore the behaviour of polyionic species, such as poly(phosphoester).

The chemical identity of the hydrophobe is also important. The more strongly hydrophobic siloxane and perfluoroalkane were both found to be highly effective. These more exotic materials, relative to hydrocarbon based surfactants, have potential to preferentially interact with surfaces of related energy. This was observed here when Silwet L-77 provided better resistance upon a hydrocarbon SAM than a perfluorocarbon SAM. It may then be expected that the Zonyl FSO-100 surfactant should perform most effectively upon the perfluorodecane SAM but it performed comparably upon both hydrophobic SAMs. So, the chemical nature of the surface and surfactant may benefit from being of related electron density (e.g. to maximise van der Waals [161]) but it is important that, should one material be more hydrophobic than the other, it must be the surfactant moiety which is the more hydrophobic of the two.

**Mechanism of surfactant resistance:** The surface tension of a liquid water interface is induced by the presence of a contiguous and extensive phase separating material (be it gas, liquid or solid). This drives protein adsorption at the aqueous phase boundary. Indeed, proteins demonstrate a generic surfactant activity reducing water interfacial tension [162]. This being the case, the mechanism of surfactant mediated resistance is likely to be two-fold; an excluded volume effect resulting from the hydrated PEG chains coating the surface in high density, and the abatement of surface tension through the dehydration of the interface and equalisation of the hydrogen bonding propensity of water proximal to the surface to that in the bulk. The former is a general mechanism [92] and one of broad acceptance, but the latter is likely a more specific property of a self-organising surfactant film which has been engineered to obviate interfacial tension.

The surfactants adsorb to the interface and so exclude other solutes from it, e.g. serum components. Pre-exposure of the surface to surfactant containing buffer allows equilibration and dense coatings of surfactant to be accumulated. Also, diffusion constants of low molecular mass surfactants will be greater than larger solutes (e.g. albumin) so should they elute from the surface it is more likely that a surfactant
molecule will repopulate that location. Any chemical functionality which interacts strongly with water will be able to perform this function to some extent [92] and all surfactants reduced the extent of protein fouling to some degree. This effect alone cannot explain the resistance of the surfactant interfaces or covalently stabilised interfaces presenting the same functionalities (e.g. the OEG-SAM presented in Chapter 4) would achieve the same level of resistance as the resistant PEG surfactants reported here. The quality of surface hydration is clearly important. As the analogous non-covalent surfactant C_{12}E_{6} is anticipated to self-organise into hemi-cylinders upon hydrophobic surfaces [160], and the OEG-SAM is planar (having been assembled in an anhydrous solvent) they may both be regarded as being suboptimally hydrated. The OEG-SAM may therefore be anticipated to foul, as observed in Chapter 4, as would C_{12}E_{6} observed here.

The principle difference between covalent and non-covalent interfaces is that the when an experimentalist deposits a covalent film they must make assumptions about many variables. For example, the solvents used to deposit covalent coatings are typically organic solvents and it is perhaps simplistic to assume surfaces deposited in one solvent may operate optimally in another. In contrast, surfactant coatings are adaptive and continuously respond to changing conditions to minimise the surface energy of the interface. The presence of an interface typically results in the distortion of hydrogen bond networks creating hanging hydrogen bonds across the interface. A spectroscopic study of interfacial hydration of surfactants L-77 and C_{12}E_{8} demonstrated that an interface coated with the siloxane surfactant L-77 had no detectable hanging hydroxyls, in contrast to the alkyl surfactant C_{12}E_{8} [163]. The hydrophiles of these surfactants are both PEG and of similar length and so it is the structure of the hydrophobic functionalities which creates this effect. In the serum fouling titrations presented here L-77 was found to foul to a lower level relative to C_{12}E_{8}. C_{12}E_{8}, as with C_{12}E_{6}, is thought to assemble into hemi-cylinders upon the hydrophobic interface [160]. This suggests that water molecules, wherever they are in the coating, may have an energetic consequence to the wider system with the invoked surface tension propagating toward the bulk solvent.

The hydrogen bonding of a single water molecule can be cooperative or anti-cooperative depending upon its engagement with surrounding water molecules. It is therefore possible that water confinement may have energetic consequences which propagate through the hydrogen bonds of the water network creating surface tension across the transient frameworks formed by the hydrating water. The coordination of BSA upon an OEG-SAM interface has been reported [135] and suggests that, though such coordination was reversible, this hydrated surface does not abate surface tension sufficiently to completely prevent protein coordination and there remains an energetic advantage to dehydrate the interface and equalise the propensity and capacity of interfacial water to engage in dynamic hydrogen bonding akin to bulk water. The slight serum fouling observed here upon C_{12}E_{6} may be equally explained and thus the method of deposition of this self-organising brush (i.e. covalent thiol stabilised self-assembly upon gold or non-
covalent self-assembly upon undecanethiol) appears less relevant. However, though the fouling observed upon the surfactant interface is in the same order as the covalent interface, it was exposed to undiluted animal serum as opposed to 1 mg.ml$^{-1}$ purified buffered protein preparations. So, the surfactant coating may be expected to be more effective as resisting fouling. It would be interesting to more directly compare these surfaces in reciprocal adsorption testing.

The surfactants assemble upon with the interface to minimise its free energy. They present a physical barrier to protein adsorption. However, they are also a scaffold on to which a three-dimensional framework of water may be assembled, as has been proposed to explain the protein resistance of the OEG-SAM [164]. But within a surfactant film these are not static entities, they will be variably responding to the thermal processes of the bulk solvent. As such they will be dynamically engaged with solvent entropy which drives biological processes. There may be a role for translational entropy, too, as surfactants may laterally migrate across the planar interface. This will increase entropy which is anticipated to further reduce the energy of the interface. The surfactant films are non-covalent and this allows the water to dynamically explore conformations to mature the self-assembled coating to minimise its energy. This is ultimately dictated by the structure of the surfactant, and chemical nature of the interface upon which it is adsorbed, but should the right surfactant be supplied to the interfacial system the surface tension of interfacial water may be negated and all fouling of protein upon the interface may be abated. This is exactly the kind of interface required for real-time monitoring of biohazards using affinity biosensors, such as SPR based sensors.

**Resistant by design:** Taking a broad perspective across all the materials tested, the following observations are made concerning the properties which are likely to make total protein resistance possible;

- **The physical nature of the surfactant hydrophilic moiety:** Pendant hydrated polymer chains must form a brush of sufficient density to prevent the penetration of problematic molecular species. These species will vary with application but for environmental monitoring these may be assumed to be very widely heterogeneous and fluctuating in concentration. The requisite density will likely be a function of the net concentration of such species and their physical size (specifically focusing upon the smallest species for scale and then generalising for concentration of all solutes as it likely relates to the water activity of the net system). These observations are in-line with theoretical prediction and relate equally to covalent interface design [152]. It may be advantageous that polymer be dynamically reconfigurable to enable the hydrating water to engage with thermal processes within the bulk solvent (e.g. mirroring system entropy). This putative facet of polymer behaviour will be better supported by non-covalent surfaces.
• **The chemical nature of the surfactant hydrophilic moiety:** Polyethylene ethers have been shown to be particularly useful. They are unlikely to be the only suitable chemistry, but the rotational entropy supported by the ether bond is high and the cis orientation of the hydrated chain integrates well with the hydrogen bond framework of the surrounding water, primarily as a result of the ether oxygen. Alternative chemistry could function in the same way but the balance of properties observed in PEG/OEG may be unique.

• **The physical nature of the surfactant hydrophobic moiety:** The surface adsorbed hydrophobe must provide enough lateral space for the hydrated pendant chain to freely interact with the aqueous solvent, though not compromise the requisite density to physically exclude any partitioning species, e.g. peptides. If the lateral density of the surfactant is too high the solvation of the pendant chains will be compromised promoting adsorption upon the brush. Even though this may be readily reversible and weak in nature any such interaction would reduce the functional performance of real-time, on-line affinity biosensors. Complete resistance is necessary. Upon planar surfaces this is possible where the coating is uniform and two-dimensional, i.e. lacking nanoscale features such as hemi-cylindrical micelles.

• **The chemical nature of the surfactant hydrophobic moiety:** The hydrophobe employed must be efficiently excluded from the solvent (i.e. must have a high surface activity). The role of lateral translational entropy is uncertain but this may augment system behaviour.

• **The chemistry of the underlying surface:** The surface upon which the surfactant coordinates should mirror the surface energy of the hydrophobic moiety to optimise the enthalpy of interaction. It should not be of a lower energy than the surfactant as this may promote surface exchange of surfactant with other solvated species.

**Kinetic vs. Thermodynamic protein fouling resistance:** The above, as a concerted whole, may lead to a thermodynamically stable brush which will be persistent, self-cleaning, self-healing, and even adaptive to changing environmental conditions (e.g. temperature fluctuations). For example, the antibody which was applied to the methyl terminated SAM was effectively cleaned from this surface by Zonyl FSO-100 (Figure 5-17). So, protein adsorption upon the surface, or the surfactant film, will be energetically unfavourable. Such a claim requires further validation, e.g. extended incubation of dynamic interfaces with fouling species for tens of hours.

In Szleifer’s single chain mean field model solvent behaviour is approximated [165]. This is the only approach to integrate solvent behaviour. The model suggests that covalently tethered polymer chains may either provide kinetic resistance where fouling will be inevitable, or thermodynamic resistance where it would occur but the extent may be suppressed [153]. A solvated chain with affinity for the surface would
reduce the density of fouling by eroding the thermodynamic benefit. Conversely, a chain with greater affinity for the solvent would present a kinetic barrier to surface adsorption. The surfactants do both. They have one chemical block with surface affinity and one with solvent affinity. So, penetration of the brush is kinetically improbable and displacement of the brush is thermodynamically unfavourable. So, assuming free surfactant can be maintained in the bulk aqueous phase, the surfactant systems are thought to enable thermodynamic resistance.

**The fouling resistance of polyethylene ethers is context dependent:** The integration of non-fouling tethers into the surfactant coating was thought to be a simple task, the tether having the same chemical identity present in the surfactant. However, while the integration of the tether was simple it fouled non-selectively. This can only be as a result of the local environment of the PEG and suggests that hydrated PEG is actually capable of interacting non-specifically with biological materials and that this may be more pronounced at phase boundaries. Such boundaries herein were the solid-liquid phase boundary at the interface but also the protein-solvent interface of the streptavidin.

The tethers themselves are found to foul proportionately, and therefore fouling is likely occurring directly upon the oligoethylene tethers. This indicates the fouling resistance of oligoethylene is context dependant, i.e. sterically constrained oligoethylene may be predisposed to adsorb protein non-specifically. This is the first report of protein fouling upon oligoethylene where an exogenous force (e.g. mechanical pressure [166] or desiccation [106]) has not been applied and the fouling which is observed may be directly ascribed to the PEG. The observation that entropically constrained PEG/OEG is a hot-spot for non-specific interaction is only possible due to the astonishingly successful fouling passivation provided by the self-organising oligoethylene glycol surfactants.

In a broader context such an observation would help define the mechanism by which “PEGylation” extends the circulation time of therapeutic agents *in vivo*. The mechanism by which this occurs is not yet fully defined. Current theories, based upon increased hydrodynamic radii of PEGylated products, do not fully account for the pharmacokinetics observed. The increased propensity of interfacial PEG to interact non-specifically with serum proteins may help to explain this effect more fully. This would be ironic considering the broad expectation that the polymer resists non-specific interaction.

**Integrating biological recognition elements:** The streptavidin applied here has retained activity within all the buffers tested (specifically; Zonyl FSO-100, Triton X-100 and Silwet L-77 all at a concentration of 0.05% mass.volume⁻¹). Streptavidin is a particularly robust protein but the antibody adsorbed to a hydrophobic SAM retained activity, too. Biological entities, therefore, maintain their structural identity within the
surfactant systems tested, at least for the duration of the tests undertaken, though these were not extensive tests.

While proteins retain their function the tether used must be reengineered. The tethers tested here appear to have introduced hot-spots for protein fouling. The antibody directly adsorbed to the interface demonstrated little obvious serum fouling and so this antibody demonstrated fouling resistance to animal serum constituents, it just needs to be covalently linked to the surface and embedded within the surfactant coating. So the issue may be overcome but it presents a manufacturing challenge: to maintain the integrity of the antibody through the process of deposition. There is scope to integrate synthetic recognition elements (e.g. “SOMAmers” [50]) which would make this much more convenient. Such a development would also likely enable the use of detergents to periodically clean the surface.

Even is the issues of fouling to the surface, and integrating a recognition element are resolved there may well be a future issue of fouling to the recognition elements (e.g. antibodies) from non-biological environmental detritus, e.g. soil and soot particulates. This issue may be anticipated but with a surfactant system it should be possible to re-formulate or blend the surfactants to mitigate any such environmental material. This is a most attractive potential benefit and, should a certain soil type be prevalent within a specific environment, it may be possible to engineer a niche surfactant system to mitigate it [167]. The possibility of using different phases within the surfactant system may help here where a perfluorinated surface/surfactant pair is used to maintain fouling resistance of the surface while an energetically distinct alkyl particulate/surfactant system may be developed to prevent soil/soot fouling to the recognition elements. The surfactant system may, in principle, be reengineered and reformulated to meet the variable needs of different applications.
6. **General Discussion and Future Work**

In principle, affinity biosensors could be used to continuously analyse environmental biological entities, continuously collected by air samplers, in near real-time. In so doing we may detect and identify specific materials of concern (e.g. toxins, pathogens, irritants etc.) present in the air we breathe, in order that we may avoid or minimise our exposure to them. The specific benefit of affinity biosensor is that they can be, at the point of analysis, reagentless. All that is required to exploit these technologies is a sensing interface which binds only the materials of concern and rejects any and all other materials. The concept is simple but such a goal is beyond the achievement herein.

However, significant progress has been made. Two principle outcomes are summarised below followed by a unifying narrative which correlates observations throughout this body of work. Finally, future work is presented which may lead to the realisation of real-time affinity biosensor monitoring of environmental biohazards and broader benefits in bioaffinity research.

Recombinant antibodies may now be routinely chemically engineered, at the point of manufacture, to facilitate their controlled and reproducible integration into technologies, such as biosensors. The “PDI” tag (reported in Chapter 3) is a generally applicable fusion peptide providing site-specific chemical functionality which is intrinsically non-toxic to microbial expression hosts. The benefit of using this tag is specific to periplasmic expression systems (though it is anticipated to be equally applicable to yeast expression, e.g. within the endoplasmic reticulum). When the PDI tag is expressed in the cytoplasm\(^4\) the benefit of the tag is lost. So, the behaviour of the tag appears to be context dependant: when oxidised/constrained it is soluble but when reduced/open it is not. Should this be the case, it is an interesting physicochemical switch of consequence to the broader conclusions of this thesis.

For the scFv scaffold the PDI tag is of utility but the current drive for recognition element engineering is to make them more inherently robust, e.g. “Adhirons” [168]. The inherent thermodynamic stability of such scaffolds means the in vivo constraints upon their chemical engineering are different. They do not require periplasmic expression and can be manufactured through more conventional cytoplasmic methods. In addition, ongoing development of unnatural amino acids (with requisite engineered expression hosts) makes it possible to readily integrate non-native chemistry within recombinant materials [169]. Such approaches widen the chemical palette beyond the constraints of native amino acids. These concomitant developments will likely obviate the need of the PDI tag in the long-term.

\(^4\) Admittedly inadvertently! The rapidly folding shark domain antibody “5A7” was found to fold before it could translocate from the cytoplasm to the periplasm. This observation has been made of other single domain antibodies.
While such parallel developments continue it will be more appropriate in the short-term to apply the PDI tag to the expression of domain antibodies (i.e. cameld or shark immunoglobulin domains) within eukaryotic expression hosts, such as *Pichia pastoris*. In such a host the nascent proteins are directed into the oxidising environment of the rough endoplasmic reticulum at the point of synthesis. Here the PDI peptide loop will fold appropriately and is thus anticipated not to compromise the solubility or yield of the product. The resulting materials will be thermodynamically stable and the PDI tag will allow simple covalent integration into technologies.

Unfortunately, in this work, the interface upon which the PDI tagged domain antibody “5A7” was to reside did not emerge from development (through Chapters 4 and 5). Had it done so it would have been interesting to compare the benefit of its orientated immobilisation. However, it was anticipated that the OEG tether would serve to support rotational behaviour of the tethered protein and so the explicit benefit of orientation may be reduced relative to solid planar interfaces. It is likely, though, that the reproducibility of immobilisation using the tag would be greater, especially when multiple antibodies are to be immobilised, in an array and some of which may present lysine residues within their CDRs.

**Non-specific protein adsorption to the solid-liquid interface may now be completely inhibited, or at least undetectable through SPR (and thus below a density of 5 µg.m⁻²).** The level of fouling resistance observed in Figure 5-10 (where Zonyl FSO-100 is partnered with a alkyl terminated HPA Biacore chip), has not been observed for such a thin coating. Comparable claims have been reported for poly(carboxybetaine) polymer brushes [154]. But there are some considerable differences between this reported surface and the simple surfactant system employed here. For example, the surface is an ATRP brush 15-20 nm in depth, in the dry state. This erodes the sensitivity of the SPR system roughly by 25% (as judged by the variance in the bulk refractive index changes attributable to the whole plasma injected). Blocking agents are used before and during the binding test (e.g. SuperBlock™ and BSA in the running buffer at 1 mg.ml⁻¹). In addition, the sample tested was plasma with anticoagulants present (acid-citrate-dextrose) which abound with carboxyl groups, the very functionality the surface coordinates the solvent with. So, in comparison to this rather elaborate system, a molecular monolayer less than 5 nm thick in the hydrated state, without need of blocking agents etc. is much more controlled and more simply prepared.

In order to apply this interface as an affinity sensing interface antibodies were successfully immobilised upon the poly(carboxybetaine) carboxylic acids. Perhaps an elaborate blocking routine will be required to passivate the surfactant interface should antibodies be integrated? But there is indication that this may not be the case (Figure 5-17). So, the surfactant system offers great potential to be an elegant, simple and more effective approach.
The power of the surfactants is surprising. Surfactants are broadly considered to play an indirect and auxiliary role in reducing non-specific binding in biosensor applications. As such their capacity to directly mitigate non-specific binding has not been widely reported (though it has been indicated in unrelated applications (e.g. plant photosystem assembly upon hydrophobic interfaces [170], protein capillary electrophoresis [171], and functionalisation of carbon nanotubes [172]). This is surprising and appears a potentially transformative opportunity which has been hidden in plain sight by the assumptions of both theoretical and practical scientists alike. This work demonstrates that simple fouling resistant interfaces are a credible research goal, something scientific funding bodies and scientific practitioners alike had abandoned as practically unlikely [151]. The observation that surfactant films are capable of resisting fouling from whole serum will prompt further investigation of the physicochemical mandate of such coatings. This will then provide more defined design requirements for fabrication methods which has been lacking.

The novelty of the surfactant interface comes from how it is manufactured more than what is manufactured. For example, the construction of a hydrophilic polymer brush is a well-known theoretical concept to prevent surface adsorption of proteins [93]. But most synthetic chemical techniques employed to physically construct these theoretical models (e.g. those used in Chapter 4) require the use of solvents alien to proteins and the intended application. Considering water is the universal biological solvent, and fundamentally drives much biological behaviour (e.g. protein folding, enzyme function, membrane partitioning etc.), the assumption that solvent substitution will not be of any consequence is profoundly hopeful. So, the novelty in this work is that the surfactant interfaces are self-assembled in an aqueous solvent and thus the resulting fouling resistance is a direct consequence of waters thermodynamic interfacial processes which have been specifically invoked to organise these nano-engineered coatings and use them as an energetic scaffold to optimise hydrogen bonding and thus minimise interfacial tension.

The Spencer group developed a philosophically related non-covalent interface [173] based on poly lysine with pendant PEG chains. The “bottle brush” polymer was then electrostatically bound upon negatively charged interfaces, such as niobium oxide (Nb$_2$O$_5$) and tantalum oxide (Ta$_2$O$_5$). These interfaces were assessed for their intrinsic resistance to human serum [174] and fouling was below the sensitivity limit of the optical biosensor employed (OWLS). The principle difference between this system and those tested here is the length of the PEG employed. In this system, human serum adsorption was undetectable when the monomer density of ethylene glycol was ≥20 nm$^{-2}$ and the shortest PEG length was 1 kDa (approximately 22 ethylene repeats) [175]. Due to the use of electrostatics in surface assembly, ionic strength of the overall solvent system was of consequence to protein fouling behaviour [176]. The advantages of the surfactant systems investigated here are that the length of the PEG chains is shorter and
the molecular entities, and the surface to which they bind, are non-ionic. This should make these interfaces more versatile in the samples they may interrogate, and the sensors they may be applied to (e.g. electrochemical systems). Interestingly when the Spencer interface was modified to carry biotin groups upon the terminations of the PEG chains, serum resistance was maintained, but when streptavidin was applied to the interface fouling was once more observed [177]. The tethering point upon the surface did not foul and so it appears possible to shroud the surface immobilised tether to inhibit fouling. However, in the surfactant system the surrounding coating is more dynamic and so it is likely that the “interphase” water will endure as the surfactants are more disordered than the immobile poly-lysine appended PEG in the Spencer interface. Another important point is that the immunoglobulin immobilised upon the streptavidin did not increase the extent of fouling. This is a good indication that biological fouling to this class of recognition elements is unlikely, at least from animal serum.

The surfactants which promote resistant are known to reduce the interfacial tension of water to a considerable degree. Indeed, the most efficient and effective of these, Zonyl FSO-100, reduces the surface tension of water most of all. Surface tension is not, however, the defining principle by which these surfactants operate. The work of Roach et al. clearly demonstrated this when they compared surfactants for use within a bioanalytical microfluidic system [178]. Several surfactants were compared therein and two of these perfluorinated surfactants are of relevance here; an ethoxylate (Zonyl FSO-100) and a simple hydroxyl. The surface tension afforded by these two surfactants was reportedly 18 and 13 mN.m\(^{-1}\) for the ethoxylate and hydroxyl species respectively. An emulsified protein solution, within a perfluorinated oil, was prepared using each surfactant and (through the monitoring of fluorescent labels) protein was found to aggregate at the phase boundary when the hydroxyl terminated surfactant was employed, whereas a stable distribution of protein was maintained when the ethoxylate surfactant was employed. So, the physical three-dimensional nature of the brush is essential to resist protein fouling and the fact these surfactants reduce the surface tension is probably a necessary supplementary property to prevent the moderate fouling observed in the less resistant but physically analogous surfactants (e.g. C\(_{12}\)E\(_6\)). The fact the perfluorinated hydroxyl surfactant adsorbs protein also supports Vogler’s concept of fouling mediated by “interphase” water discussed in Chapter 4: water, bound upon an interface, is going to be entropically compromised and this cascades through the hydrogen bond network to influence other water molecules, and other entities in the system may readily displace this water network. So, any two-dimensional interface may therefore expect to foul to some extent whether covalently or non-covalently fabricated, such as the carboxyl terminated monolayer of the C1 surface reported in Chapter 4 (which, peculiarly, adsorbs 1% of a protein monolayer regardless of the molecular mass of the adsorbing species) or the perfluorinated hydroxyl surfactant.
In summary, the key determinant of fouling resistance appears to be a three-dimensional entropic brush composed of PEG/OEG which is self-organising upon a hydrophobic interface and which minimises the interfacial tension of the system through optimised physical and chemical interactions with water and the surface. However, while such an interface holds promise for affinity biosensor applications, it will only be useful when a recognition element can be covalently integrated within it without reintroducing fouling hot-spots.

**Why Surface Plasmon Resonance?** The interface developed using SPR should find broad application. There is nothing about it which will limit its application as, for SPR, these coatings have simply been a passive enabler, i.e. they have no niche role in transduction. The interface could be applied to all affinity biosensors including electrochemical sensors. The electrode types are broad, glassy carbon, graphene, gold etc. Electrochemical passivation of the electrodes will occur to a variable extent (e.g. partial passivation for Triton X-100 [179], and near complete for Zonyl FSO-100 [180]) but through the engineering of the surfactant entities it should be possible to integrate redox functionality to work as an electron mediator. The system should, therefore, port relatively well onto other sensing platforms. The exception may be mechanical sensors such as the QCM and cantilevers. The rheology of surfactant coatings may cause issue as, for example, L-77 has been found to have sheer thickening behaviour. The phase behaviour of the surfactants is variable and so complex rheology will likely limit some applications. However, there will likely be some surfactant system which could be co-opted or reengineered to suit.

Considering the likely inevitability of fouling upon affinity sensing interfaces (owing to the probable incompatibility of maintaining entropy while immobilising lumps of protein) simple mass sensing (by whatever means) is perhaps unlikely to give the level of analytical fidelity required of bioanalysis. Confirmatory reagents could be used in the same analytical cell and confirm the analysis through an alternate means, e.g. through electrochemiluminescence. The consequence of fouling is then easily mitigated and the surfactant system should maintain the viability of the electrodes and facilitate electrochemical processes by enabling the electrochemical entities to situate within a few nanometres of the electrode. Also, the interfaces are uncharged. Commercial surfactants are likely to have a good affinity for electrode materials, such as graphene [181] and so the commercial technologies readily available should be versatile enough.

**A unifying perspective – polymer-solvent interactions at interfaces:** All entities within a biological system, whether natural or otherwise, are acting upon each other and dynamically evolving system behaviour. As such, changes in one component have consequences for the others and so the system should be regarded as a “soft matter system”. For example, the context dependent behaviour of PEG/OEG: When free as a gyrating solvated chain PEG/OEG is resistant to non-specific fouling. However,
when appended to another entity in the system (e.g. the two-dimensional surface of a technology component or a protein) the entropy cost of such constraint drives non-specific interactions. This theory may equally apply to the polypeptide expressed in *E. coli* (Chapter 3). The solubility of the PDI tag is also thought to be context dependant: in its reduced, linear form the benefit it provided as a soluble, stable fusion tag was lost.

It is possible that the physical behaviour of PEG and peptide share this common mechanism. The chemical potential of water coordinated to a polymer chain may be proportionate to the disorder of the chain. Should the polymer be appended to an interface (i.e. a solid phase or a protein, e.g. planar gold or streptavidin respectively) the resulting chemical potential creates a hot-spot for non-specific protein fouling. Szleifer predicted the immobilisation of PEG would introduce inhomogeneities in the hydrogen bonding of the solvent [182]. Such inhomogeneity may therefore be expected to propagate through the three-dimensionally coordinated solvent around the polymer chain increasing the chemical potential (i.e. surface tension) of the local solvent. If this physical mechanism does indeed drive deleterious adsorptive behaviour in biological systems then the implications may be profound for many important biological issues (e.g. degenerative diseases where protein aggregation is implicated).

In support of Vogler’s protein fouling “interphase” mechanism [151] the molecular identity of adsorbing proteins upon zwitterionic and non-ionic brushes is reported to be related [183]. Proteins with native physiological behaviour which operate at phase boundaries (e.g. low-density lipoprotein [184] and clusterin [183]) are enriched within polymer interfaces where the polymer chains are accessible (i.e. convex interfaces, e.g. nanoparticles). In this context, the physicochemical behaviour of the hydrated polymer may again moderate the behaviour of hydrating water networks creating a distinct phase (i.e. water surface tension locally increases) and, on the length-scale of a linear molecular polymer, this presents a generic physicochemical cue to partition protein species evolved to engage non-specifically in such environments to prevent aberrant non-specific interaction and preserve the integrity of the broader biomolecular system (e.g. lipid transporters and molecular chaperones).

The expectation then is that any pendant ligand upon the polyethylene ether tether will trigger fouling upon the tether. Should the PDI tag, therefore, be used to immobilise recombinant antibodies upon the self-organising surfactant interface fouling would persist as the PDI loop would be reduced and therefore linearised. Based on the tags behaviour *in vivo* this linear peptide may be anticipated to foul at the junction with the core protein scaffold. In addition, conjugation of one or both of the cysteine residues to surface tethers would create further fouling hot-spots. This is what happened with the streptavidin when it was immobilised upon the OEG$_{23}$ tethers (Figure 5-26). The Spencer group also reported fouling upon a similarly functionalised surface [177]. Indeed, all aqueous phase linear polymers may respond in a similar
Further corroborating evidence comes from the broad observation that nonspecific binding in bioaffinity panning for the development of synthetic ligands (whether composed of nucleic [185] or amino acids [186]) is exacerbated when the putative ligands are presented in high density though the mechanism remains ill-defined. The fouling observed in this work increased with tether density, likely a function of additive nonspecific interactions. In addition, neutron reflection studies of protein interactions with PEG brushes have determined that fouling to the interface which the tethers are appended to freely occurs [187]. In these studies it is assumed that the fouling is occurring to the surface rather than the tethers but in neutron reflection this cannot be distinguished. It would be interesting to characterise the surfactant/PEG tether surface with pendant streptavidin using neutron reflection to profile adsorption locations and thus confirm the location of protein fouling within the tether interface.

The evidence across broad areas appears to suggest that linear hydrophilic polymers present a generic behaviour in aqueous solvents and create potent hot-spots of aberrant biological interaction when they are immobilised, and that this behaviour is mediated by solvent surface tension which develops through geometrically coordinated water.

**Developing dynamic soft matter systems – a tangible position to build from:** It’s not currently possible to create such systems from scratch due to our inability to account for water behaviour in complex systems but the use of surfactant interfaces would be a tangible starting point to learn how to make components that assemble to form functional arrangements in complex environments, like whole serum.

To this end future work may be directed in the following regards (illustrated by Figure 6-1):

- **Validate the theory of polymer fouling as a function of local entropy loss:** This is a potentially critical and universal property of solvated chains. The implications of which would be of consequence to the study of all specific and non-specific molecular interactions alike. Magnetic resonance spectroscopies may be useful in further study, such as electron-spin echo envelope modulation and Overhauser dynamic nuclear polarization can probe nitroxide spin labels (as applied to the study of protein folding within GroEL [188]). These remotely addressable labels can inform on local water density and translational dynamics. The addition of a spin label to a hydrated chain in a series of increasing separations from an interface (e.g. with a protein/polymer bead), would provide an interesting series of data points with which to profile the relative disorder of the solvent with range.

- **Characterise the relative fouling behaviour of other solvated polymers:** The context dependent fouling of PEG/OEG could only be observed due to the zero background fouling achieved with the new surfactant interface. It would be interesting to apply other well hydrated polymer chains with
and without pendant nanomaterials to understand their propensity to switch adsorptive behaviour. Of particular interest; native species (DNA as both single and double stranded entities, and peptides), synthetic species (e.g. poly(caboxybetaine) and other such materials) and hybrid species (e.g. modified nucleic acids used as synthetic ligands, such as SOMAmers [50]). Should any alternative polymer be chemically resistant to fouling (they are unlikely to be physically resistant) then they may be exploited as tethers to integrate the recognition element. Alternatively, they may be employed as synthetic recognition elements (e.g. aptamers) which would then trivialise the integration of biological recognition elements into the dynamic surfactant layer as chemical cleaning processes (e.g. SDS treatment) may be employed in surface preparations.

- **Application of the surfactant interface to other affinity biosensor technologies:** SPR has been used to develop the surfactant interface. The design principles may be easily ported to other platforms, for example, electrochemical biosensors. While the use of a material like Zonyl FSO-100 is likely to passivate electrodes (rendering them insensitive due to the probable high density and defect free nature of the coating [180]), it may be possible to specifically engineer surfactants to integrate redox activity and thereby restore electron transfer. Indeed, the ethoxylation of anthraquinone has already been reported [189]. The hydration behaviour of these redox species, as they charge and discharge, would need to be characterised as they may inadvertently draw solvent into the surface and destabilise the resistant films. So, this may not be a trivial chemical engineering strategy. Figure 6-1 depicts a functionalised Indium Tin Oxide interface which may be used to drive the nano-scale electronics within the aqueous system above. Nanoparticles are routinely manufactured using non-covalent means to chaperone and derivatise solvent suspensions of nanoparticles [190]. The depiction in Figure 6-1 is fantastical but if non-specific fouling cannot be abated directly, as may be anticipated, then secondary reagents will be necessary to confirm the identity of adsorbed species, potentially following a related approach. The fusion of mass sensing modalities with electrochemical modalities could yield a very sensitive and specific interface.

- **Explore alternative solutions to integrate a molecular tether into the surfactant layer:** Regardless of the transducer function the tether fouling issue will benefit from further work. It may be possible to construct a tether which possesses sequential chemistries that mirror the phases it must pass through as it moves from the gold surface, through the hydrophobic SAM, through the hydrophobic component of the surfactant layer, and finally out into the PEG/OEG brush. By mirroring the local chemistry the physical issue of compromised entropy may be enthalpically abated. This would only resolve the issue on one end of the tether, to prevent fouling at the end which binds the recognition element would require an alternative solution. Shrouding the
recognition element within the OEG/PEG brush is an option but this would make the immobilisation of the recognition element more challenging (unless they are intrinsically resistant to detergents such as SDS, e.g. synthetic recognition elements or potentially Adhirons which, if not resistant, may refold).

- **Explore the interactions of difficult to study molecular species:** The novel surfactant interface may find broad utility in the study of affinity interactions in complex sample types. This would be contingent upon inserting a ligand into the coating. However, the study of intrinsically disordered proteins (IDPs) is difficult within the physiologically complex environments in which they function. The surfactant interface could allow the study of IDPs in complex matrices such as simulated cytoplasmic conditions where water activity is much reduced and solvation dynamics will change dramatically. Equally the development of medical therapeutics for degenerative protein aggregative diseases could be supplemented through the study of mechanistic triggers and progression inhibitors. This latter application may not require a fouling resistant tether to append the physicochemical aggregation trigger to.
Figure 6-1. Proposed soft matter system for biological detection and diagnostic applications. Components are numbered and a brief description provided; 1) The material interface can theoretically be any transduction component (e.g. indium tin oxide, graphene, gold, silicon etc.), 2) The surface is treated to be hydrophobic and its surface energy tuned to selectively phase separate surfactants of related surface energy (e.g. siloxane), 3) The surfactant system is engineered to promote detergency to make the surface self-cleaning but non-denaturing within biological systems, 4) The solvent rheology/phase behaviour of the surfactant formulation can be modified, if required, through buffer additives e.g. β-cyclodextrins, 5) Sample contaminants can be passivated using the same principles as the dynamic surface, 6) Recognition elements may be used to construct a sandwich assay (e.g. saccharides, proteins etc.), 7) The nanoparticles can be engineered to provide whatever function the transducer requires (examples include quantum dot/electrochemiluminescent and paramagnetic/giant magnetoresistive transduction mechanisms), 8) any nanoparticle actuating reagents (e.g. electron mediators for quantum dot luminescence) may be supplied in quantity and specifically targeted to the nanoparticles via phase separation through an appropriate surfactant phase system (e.g. perfluorinated nanoparticle/micelle system).
Appendices

Appendix A: Customisation of pAK300 vector from ref. [107]
The original pAK300 vector was modified to incorporate the restriction sites listed below. This was to facilitate the modular substitution of C-terminal fusion tags listed in Table 3-1. Coding DNA between SfiI and HindIII is substituted with the sequence below:

```
GGCCTCGGGGGGGCGGTGTTCTGGTGAGCTCGGGCTAGCATACTTTGTAGCCAGGGTAGCTGTGGAATCTGCAGCATCATCATCACCATCATTAAAGCTT
Red = SfiI    Amber = NheI    Blue = PstI    Green = HindIII
```

Appendix B: Antibody protein sequences
Sequences listed as a processed post-translational product using the E. coli expression host with an illustrative fusion tag coloured green (the protein disulphide isomerase tag in this instance). For the sequences of the alternative fusion tags applied see Table 3-1. Murine derived scFv are composed of two variable domains linked with a serine/glycine peptide. The camelid derived antibody is composed of a single variable domain.

**scFv OvE**
```
DYKDIVMTQSPASLAVSLGQRATISCRASQSVSTSSYSYMHWYGQPKGQQPKLKKLYSNLESGVPARFSGSGTDFTNL
IHPVVEEDTATYYCQHWSWEIPFTFGGTTKEIKRGGGSGGGSGSGVEKLMESGGGLVKGPGGLKLSCAASGFTSSYAMS
VRQTPEKRLEWVATIRSGLGTYYYPDSVKGRFTISRDNAKNTLHQMSSLRSEDTAMYCARHRMITTAGDAMYVGQ
GTSVTVSASAGAGGGSAGAPASNCRSQGSCWNLQHHHHHH
```

**scFv OvA**
```
DYKDIVLTQSPASLAVSLGQRATISCRASQSVSTSSYSYMHWYGQPKGQQPKLKKLYSNLESGVPARFSGSGTDFTNL
IHPVVEEDTATYYCQHWSWEIPFTFGGTTKEIKRGGGSGGGSGSGVEKLMESGGGLVKGPGGLKLSCAASGFTSSYAMS
VRQTPEKRLEWVATIRSGLGTYYYPDSVKGRFTISRDNAKNTLHQMSSLRSEDTAMYCARHRMITTAGDAMYVGQ
GTSVTVSASAGAGGGSAGAPASNCRSQGSCWNLQHHHHHH
```

**scFv M28**
```
DYKDIVLTQSPASLAVSLGQRATISCRASQSVSTSSYSYMHWYGQPKGQQPKLKKLYSNLESGVPARFSGSGTDFTNL
IHPVVEEDTATYYCQHWSWEIPFTFGGTTKEIKRGGGSGGGSGSGVEKLMESGGGLVKGPGGLKLSCAASGFTSSYAMS
VRQTPEKRLEWVATIRSGLGTYYYPDSVKGRFTISRDNAKNTLHQMSSLRSEDTAMYCARHRMITTAGDAMYVGQ
GTSVTVSASAGAGGGSAGAPASNCRSQGSCWNLQHHHHHH
```

**scFv M67**
```
DYKDIVLTQSPASLAVSLGQRATISCRASQSVSTSSYSYMHWYGQPKGQQPKLKKLYSNLESGVPARFSGSGTDFTNL
IHPVVEEDTATYYCQHWSWEIPFTFGGTTKEIKRGGGSGGGSGSGVEKLMESGGGLVKGPGGLKLSCAASGFTSSYAMS
VRQTPEKRLEWVATIRSGLGTYYYPDSVKGRFTISRDNAKNTLHQMSSLRSEDTAMYCARHRMITTAGDAMYVGQ
GTSVTVSASAGAGGGSAGAPASNCRSQGSCWNLQHHHHHH
```

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scFv B04
DYKDVLMTQTPLTLSVTIGQPASISCKSSQSSLDSDGKTYLNWLQSQRPGQSPKRILYLVSKLDGVPDRFTGSASHGTDFLKI
SRVEAEDLGVYYCWQGTHFPFTFGSGTKLEIKRGGGSGSGGGSGGGGGSGGGGSSGSEVQLVESGGGLVQPGSRRKSLCAAS
GFTFSSYGMSWVRQTPDKRLEWVATISSGGRYTFYPNSVKGRTFTRSDNATOMLYMQSNLKSERTAMYYCARHKTYYG
SSLDFWQGGTTLTSAASGAGGSGGAPASNRCSQGSCWNLQHHHHHH

DAb 5A7
ARVDQTPRSVTKETGESLTINCVLRDASYALGSCWYRKKSGEGNEESISKGGRYVETVNSGSKSFLINDTVEGGGTYR
CGLGVAGGYCDYALCSSRYAECGDTAVTNAASGAGGSGGAPASNRCSQGSCWNLQHHHHHH
7. References

2. Wheelis, M., **Biological warfare at the 1346 Siege of Caffa.** Emerging Infectious Diseases, 2002. 8(9): p. 971-975.


“Skadoosh”

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