Strategies for creating antifouling surfaces using self-assembled poly(ethylene glycol) thiol molecules

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This thesis has been submitted to the Faculty of Science at Aarhus University, Denmark, in order to fulfill the requirements for obtaining a PhD degree in Nanoscience. The work was carried out under the supervision of Prof. Peter Kingshott and co-supervisor Rikke Louise Meyer at Interdisciplinary Nanoscience Center (iNANO).

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ABSTRACT

Microorganisms are one of the most important parts of our ecosystem influencing the sustenance of human society. The beneficial microbes are of high relevance to food industry, development of antibiotics and processing of many raw materials. Mankind has indeed benefitted a lot from large number of microbial species, but then the environment is also teeming with pathogenic microbes that pose serious threat to human health. Hence the success of human survival not only depends on exploiting the useful microbes but also on our ability to defend ourselves against the pathogenic ones. Microbes such as bacteria can exist either in free floating planktonic form or as biofilm which can defined as adherent microbial colonies embedded in slime or extracellular polymeric substance. Microbial biofilms are responsible for a large number of problems posing a serious safety threat within our society. Biofilms have substantial impact on human health, as many bacterial infections are caused by or involve biofilms. Biofilm infections are for example often associated with medical implants, as artificial surfaces in the human body provide a safe haven where biofilms can form. The food industry daily combats biofilms forming on the surfaces of equipment to avoid contamination of their products. In addition to posing a health problem, biofilms also pose technical problems, such as corrosion and reduced water flow in technical water systems. Removal of a biofilm is very tedious and sometimes even impossible because bacteria in biofilms are resilient towards antibiotic and biocides. It is therefore desirable to be able to prevent biofilm formation, rather than attempting to eliminate biofilms after they form. The formation of a microbial biofilm on a surface can be prevented by creating unfavourable conditions for the reversible, initial attachment of microbial cells. This effect can be obtained by grafting hydrophilic polymeric chains onto surfaces and thereby provide a steric barrier between the substrate surface and the microbial cell. Poly (ethylene glycol) (PEG) is one of the most widely used polymers for making non-adhesive coatings. The work presented in this thesis involves grafting PEG chains onto surfaces using different modifications of the ‘grafting to’ technique.

The main aim of studies presented in this thesis was to develop surfaces which would prevent bacteria from forming biofilm. The work focuses on novel strategies to self assemble PEG thiol monolayers with high graft density. One of the strategies investigated involved
backfilling a self assembled layer of 2000 Da PEG thiol with shorter oligo (ethylene glycol) (OEG) thiol molecules to form a mixed monolayer. Detailed quantitative characterization of the backfilling process was carried out using x-ray photoelectron spectroscopy (XPS), contact angle measurements, and atomic force microscopy (AFM). These studies helped in understanding the arrangement of backfilled molecules and the extent of desorption of PEG molecules during the backfilling process. The PEG SAM backfilled with OEG molecules was tested for resistance towards serum adsorption and bacterial attachment, and was found to better resist fouling compared to PEG SAM that had not been backfilled.

The second strategy involved PEG grafting using supercritical carbon dioxide (SFC), which is known to possess robust solvation properties. The idea was to demonstrate ‘cloud point’ type grafting during self assembly using SFC, and produce PEG SAM of high graft density. The SFC based PEG grafting reported here is the first of its kind, and the results demonstrated that SFC is a good solvent for polymer grafting. The fouling properties of such layers were ascertained by quantitative protein adsorption studies and bacterial attachment studies. The detailed surface characterization of grafted polymeric layer and antifouling studies helped in development of novel ways to create antifouling surfaces which have the potential to counter problems caused by biofilms.
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List of Publications

Publications that form the basis of this PhD thesis:

1. **Arcot R. Lokanathan**, Shuai Zhang, Viduthalai R. Regina, Martin A. Cole, Ryosuke Ogaki, Mingdong Dong, Flemming Besenbacher, Rikke L. Meyer and **Peter Kingshott**. Mixed monolayer formation by backfilling of thiolated poly(ethylene glycol) monolayer with oligo (ethylene) glycol terminated alkane thiol *Biointerphases, submitted*

2. **Arcot R. Lokanathan**, Viduthalai R. Regina, Martin A. Cole, Ryosuke Ogaki, Flemming Besenbacher, Rikke L. Meyer and **Peter Kingshott**. Cloud point grafting of thiol functionalized poly(ethylene glycol) chains using supercritical carbon dioxide. *In preparation, to be submitted to Journal of supercritical fluids*


Publications to which I have contributed as part of the project "Nano- and bio-functionalised surfaces for biofilm prevention". These publications are not included in the PhD thesis and will not be discussed in the thesis.


Conference publications


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>AHL</td>
<td>N-acyl homoserine lactones (AHL)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
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<tr>
<td>DOPA</td>
<td>dihydroxyphenylalanine</td>
</tr>
<tr>
<td>ESCA</td>
<td>electron spectroscopy for chemical analysis</td>
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<tr>
<td>EPS</td>
<td>extracellular polymeric substance</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>HAS</td>
<td>hemispherical analyzer</td>
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<tr>
<td>LCST</td>
<td>lower critical solution temperature</td>
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<tr>
<td>Lyz</td>
<td>lysozyme</td>
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<tr>
<td>MEMS</td>
<td>microelectromechanical devices</td>
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<tr>
<td>$M_W$</td>
<td>molecular weight</td>
</tr>
<tr>
<td>OEG</td>
<td>oligo(ethylene glycol)</td>
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<tr>
<td>PAA</td>
<td>poly(acrylic acid)</td>
</tr>
<tr>
<td>PB</td>
<td>polybutylene</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
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<tr>
<td>PDMS</td>
<td>poly(dimethylsiloxane)</td>
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<tr>
<td>PE</td>
<td>polyethylene</td>
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<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
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<tr>
<td>PEI</td>
<td>poly(ethylenimine)</td>
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<tr>
<td>PEO</td>
<td>poly(ethylene oxide)</td>
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<tr>
<td>PHEA</td>
<td>poly(hydroxy ethylacrylate)</td>
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<tr>
<td>PP</td>
<td>polypropylene</td>
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<tr>
<td>PPO</td>
<td>poly(propylene oxide)</td>
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<td>PLL</td>
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<td>PLL-g-PEG</td>
<td>poly(l-lysine)-g-poly(ethylene glycol)</td>
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<tr>
<td>PMMA</td>
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<tr>
<td>pNa</td>
<td>para-Nitroaniline</td>
</tr>
<tr>
<td>PP</td>
<td>polypropylene</td>
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</table>
PU – polyurethane
PVC – polyvinyl chloride
QCM-D – quartz crystal microbalance with dissipation
QNM – quantitative nanomechanical mapping
SAM – self assembled monolayer
SPR – surface plasmon resonance
TSB – tryptic soy broth
UV – ultraviolet
XPS – x-ray photoelectron spectroscopy
INTRODUCTION

Biofouling processes on surfaces initially involve formation of an adsorbed layer (also known as a conditioning layer) of biomolecules, such as peptides, proteins and polysaccharides. This layer is speculated to facilitate attachment of microorganisms and multicellular organisms, eventually leading to biofilm formation [3, 4]. Biofilms are involved in many infections, such as urinary tract infection, respiratory diseases, dental plaques, cystic fibrosis, and failure of medical devices such as catheters, contact lenses, prosthetic joints and other implants [5, 6]. Biofilm formation is a serious problem in water distribution systems [7], reverse osmosis membranes of salt water treatment plants [8], food processing, and transportation equipment [9, 10]. Macrophouling on ship hulls increases drag, and problems caused by biofilms increase the maintenance costs in the oil industry [4]. Understanding the fundamental aspects of complex biological processes leading to biofouling would help in developing novel strategies to prevent biofouling.

The use of strategies to prevent fouling in marine applications dates back to 300 BC where sheets of heavy metal were used on wooden boats [11]. Medical applications, however, were first recorded in the early 20th century when attempts to use dental implants, artificial valves, and prosthetic bone parts started [12]. The first biocompatible polymeric material was discovered accidentally during the second world war when Ridley, an English eye surgeon, noticed that splinters from canopies of fighter planes made of PMMA inside eyes of pilots did not cause irritation [13, 14]. Systematic research on in vivo applications of natural and synthetic polymeric coatings in the field of biomedical engineering started during the latter half of the 20th century. Although marine and medical antifouling research fields developed independently until the latter half of the 20th century, introduction of strict regulations to preserve ocean ecosystems forced the marine antifouling research groups to opt for environmentally safe and non-toxic technologies similar to those used in medical research. Prevention of microbial growth by using antifouling coatings is becoming increasingly popular in food processing and packaging. The antifouling technologies used in the food industry obviously need to be non-toxic. These complex requirements for designing antifouling surfaces need an in-depth understanding of interfacial phenomena involving
interactions of biologically relevant systems with commercially relevant surfaces. During the 20th century, there were many scientific breakthroughs, such as establishment of laws and principles governing behavior of light, electrons, atoms, molecules and polymers. Consequently the last few decades of the 20th century witnessed a paradigm shift in the field of surface science (characterization, design and fabrication) that enabled the understanding of bio-interfacial phenomena at the molecular level, allowing the design of novel materials by manipulation of the chemical and physical properties at micro and nano dimensions [3, 15-17]. The advent of the field of Nanoscience, aided by advances in biotechnology and advanced analytical instrumentation made the challenging field of biomedical engineering all the more appreciable and approachable.

This thesis titled ‘Strategies for creating antifouling surfaces using self assembled poly(ethylene glycol) thiol molecules’ is a collection of studies performed as a part of my three year PhD project with an aim to develop novel ways to counter protein adsorption, bacterial attachment and eventually biofilm formation. The scientific principles behind design of strategies used in development of antifouling surfaces are discussed in Chapter 1. The project involves developing ‘grafting to’ techniques to maximize the grafting density of thiolated poly(ethylene glycol) (PEG) self assembled monolayers (SAMs) on gold substrate, the details of which are discussed in Chapters 3 and 5. Apart from using just the PEG monolayer, the possibility of using immobilized antifouling protease enzyme was investigated. The results of enzyme immobilization studies using subtilisin are discussed in Chapter 4. The thiol based SAM is one of the most popular model systems when it comes to understanding fundamental aspects of biointerfacial processes. The PEG SAMs were characterized using surface sensitive techniques; x-ray photoelectron spectroscopy (XPS), ellipsometry, and surface energy measurements. The antifouling properties of surfaces were tested using single protein or serum adsorption quantified by quartz crystal microbalance with dissipation (QCM-D), and bacterial attachment assays. The experimental techniques are described in Chapter 2. The summary of conclusions derived from all the studies performed during my PhD along with future directions are presented in Chapter 6.
CHAPTER 1: BACKGROUND

I. Mechanisms of biofouling

Microbial biofouling is a multistage process. The first stage is molecular fouling or conditioning layer formation involving the adsorption of inorganic and organic molecules on surfaces [4, 18]. The second stage is microfouling, where the biofilm is formed by attachment of single-cell organisms, such as bacteria and algae. In marine environments, the microfouling step is followed by macrofouling, which results in attachment of higher multicellular organisms such as barnacles [4, 18, 19]. The cascade of biophysical processes in biofilm formation closely resembles the processes that lead to prosthetic implant encapsulation in mammals. Once an implant is placed inside the body, proteins adsorb to the surface, followed by attachment of neutrophils, macrophages and finally fibroblasts, that encapsulate the implant with collagen [3]. The bacterial biofouling process can simultaneously occur often interfering with the success of prosthetic implants in humans. If bacterial cells succeed to attach onto an implant before encapsulation, it becomes virtually impossible to cure the bacterial infection. Although the time scale of bacterial microfouling is different from that of implant encapsulation, these two processes have very similar onset mechanisms involving conditioning layer formation followed by interactions of cells with surfaces as shown in Figure 1.

In spite of detailed studies in the field of ‘cell attachment to surfaces’ spanning over five decades, our ability to gain absolute control over the fouling processes still remains a major challenge. A reason for this may lie in the lack of detail in our understanding of how microbes attach to abiotic surfaces. Initial studies were primarily focused on the role of a few extracellular proteins or polysaccharides, but new findings in the last decade revealed that other biomolecules, such as extracellular DNA can also be crucial for bacterial attachment [20]. The biological control mechanisms involved in switching from planktonic to a sessile lifestyle is another part of the field that awaits important discoveries. Some mechanisms are controlled through quorum sensing systems, which were discovered four decades ago [21]. Until the beginning of the 21st century quorum sensing signaling molecules were thought to be
present only in a few bacterial species [21]. Now microbiologists believe that most, if not all, bacterial species utilize quorum sensing during biofilm formation, and these findings have triggered a host of studies trying to relate quorum signaling to initial attachment of bacterial cells [22]. A complete understanding of the biological and physicochemical processes involved in fouling processes will help in designing surfaces with the potential to successfully counteract biofouling. Various strategies employed to counter fouling problems will be discussed in the next section.

![Figure 1](image)

**Figure 1.** A simplified schematic representation of the stages leading to biofouling caused either by prokaryote (for example bacterial) biofilm formation or encapsulation of prosthetic implants by phagocytes.

II. Antifouling strategies:

Various strategies have been used to prevent biofouling and target specific stages during the fouling development. Broadly, the strategies can be put into the categories listed in Figure 2. Anti-adhesion coatings made by chemical or physical adsorption of hydrophilic polymer molecules work by presenting a steric and/or hydration barrier between the underlying substrate and the proteins and/or cells in solution above the surface, thereby preventing the reversible initial attachment. There are several types of coatings which are used to prevent protein adsorption and fouling. Anti-adhesive biomaterials could be of natural origin, such as dextran, BSA, chitosan, alginate, hyaluronic acid and mannitol; important
alternatives to natural polymers are organic molecules such as PEG, PEO and polyacrylamides which are prepared synthetically [13, 14, 23-30]. Zwitterionic molecules such as sulfobetaine [31] and phosphorylcholine [32] have been demonstrated to have resistance towards protein adsorption and cell attachment. The ‘cell adhesion interfering’ surface on the other hand interferes with the biological process responsible for cell adhesion, but does not kill the microbe. Post microbial attachment strategies also highly relevant in countering biofilms. The degradation of biomolecules responsible for inter cellular adhesion which hold the microbial colony together would result in detachment of cells from biofilm and eventually the removal of the entire biofilm. The studies presented in this thesis involve proteolytic enzymes immobilized onto an anti-adhesive hydrophilic coating.

**Figure 2. Classification of approaches used to make antifouling surfaces [4, 20, 33-35].**

Various physical and chemical properties of polymers need to taken into consideration in order to be chosen as candidates for a specific application. Biocompatibility, structural and chemical stability, biodegradability, glass transition temperature, charge, isoelectric pH are a few of the important properties of polymers that influence their interactions with biological environments. All the above mentioned naturally occurring polymers are biodegradable, while most synthetic polymers are often non-biodegradable. Synthetic polymers can be tailored to be biodegradable. For instance PMMA is non-biodegradable while PHEA is biodegradable, and other important examples are the development of biodegradable polyurethane [36], polylactic acid and polyglycolic acid, and their copolymers. Biodegradability of a polymer would prevent its accumulation in biological systems thereby making it environmentally safe and also degradation in response to specific stimuli such as presence enzymes capable
cleaving bonds binding the monomers could used in site specific drug delivery. It should also be noted that biodegradability could compromise the stability of antifouling coatings thereby rendering the surface incapable of resisting fouling. All of the above mentioned polymers have the disadvantage that they are not completely successful at preventing biofouling. Duracher et al. demonstrated that PNIPAM brush coated surfaces were protein resistant below its lower critical solution temperature (LCST) of 32 °C and non-resistive above the LCST, thus making it unusable at \textit{in vivo} temperatures [37]. Futamura et al and Yamasaki et al. studied coatings made of methacrylate polymeric chains terminating with phosphorylcholine groups and found that the phosphorylcholine group got embedded in the hydrophobic interior of the SAM upon drying whereas in wet conditions it was located the solvent-SAM interface [38, 39]. They observed that these surfaces once dried, needed long wetting pretreatment or prehydration (10h) times in order for the phosphorylcholine groups to reorient after reaching equilibrium, otherwise the surfaces were less efficient in resisting protein adsorption when compared to prehydrated surfaces. Therefore, the use of surfaces coated with methacrylate functionalized phosphorylcholine is not suitable for applications that cannot afford long pretreatment procedure. The ethylene glycol based polymers are prone to auto-oxidation in the presence of oxygen and transition metal ions [40]. Also hydroxyl end-groups, if present, are converted onto aldehydes by alcohol dehydrogenase, initiating further reactions that make these coatings inefficient at resisting protein adsorption [40]. Among the various types of surface coatings mentioned above, ethylene glycol based coatings such as PEO, PEG, OEG coated surfaces are one of the most widely studied, well characterized non-fouling systems which have been shown to be effective in reducing protein adsorption [41], bacterial attachment [42], and \textit{in vivo} leukocyte attachment [43].

The exact mechanism by which PEG layers resist non-specific protein adsorption still remains unclear, but there are few speculations based on detailed experimental and theoretical studies. The earliest attempt to explain the nonfouling properties of PEG was made by De Gennes and Andrade [44, 45]. They argued that when a protein molecule approaches a surface coated with PEG chains, the water molecules bound to the PEG through hydrogen bonding have to be expelled due to compression. Since expulsion of bound water molecules is energetically unfavorable, the PEG molecules do not allow the protein
molecules to reach the substrate. Although this model was partly successful in explaining systems with high molecular weight PEG, it could not explain the results of studies using oligo(ethylene glycol) (OEG) \((n<6)\) based self assembled monolayers (SAM) [46]. Whitesides et al. studied OEG systems, alkane thiol SAMs with various hydrophobic and hydrophilic end groups. Based on their studies they concluded that inertness of a surface is not only the property of hydrated polymeric layers but this could also be due to the structured water layer [47]. The structured water layer could be formed due to orientation of water molecules by dipole moments (extending over 3-4 layers of water molecules) from hydration layer at interface as suggested by Grunze et.al. based on computer simulations [48]. They also experimentally showed that the conformation of OEG was a very important structural aspect responsible for resistance towards protein adsorption. The OEG SAMs formed on Au were protein resistant, while on Ag surface the SAM was not resistant to protein adsorption. The constrained OEG molecules in SAMs formed on Ag, due to higher packing density could not attain a helical conformation, whereas the OEG molecules on Au could attain helical conformation and hence protein resistance [46]. The helical conformation enables the hydrogen bonding of water molecule with oxygen atom of polyether chain. Based on calculations using single chain mean field theory, Szleifer proposed that loss of conformational entropy of grafted polymeric chains during approach of protein molecules is responsible for the repulsive protein-polymeric chain interaction [49, 50]. Despite all the efforts to ascertain the precise mechanisms of such coatings, a universal explanation of the antifouling properties of nonionic coatings is still the subject of much conjecture and research effort. In addition, translating the theoretical findings into experimental coatings, including stability issues, has proved challenging to surface science.

Biofilms in general consist of 2-5% by volume of cells with the rest being extracellular polymeric substances (EPS) [51]. Major components of EPS are polysaccharides, extracellular DNA and glycoproteins [52, 53]. The EPS is a major obstacle in biofilm removal efforts, and it is one of the reasons for the failure of most antimicrobial strategies aimed at killing biofilms. EPS acts as a physical barrier thereby minimizing the diffusion of antimicrobial molecules into the biofilm. Efficient biofilm removal there requires much higher concentrations of toxic biocides than what is needed to kill planktonic cells [35]. Thus simple and safe
strategies to prevent biofilm formation rather than using harsh treatments to remove biofilm are highly desirable. Non-adhesive coatings made of hydrogels and hydrophilic coatings have been demonstrated to minimize microbial biofilm formation in short term experiments. Nejadnik et al. reported a coverage of approximately 80% (surface coverage) after 20h incubation with \textit{Staphylococcus aureus} on a PEO-PPO block copolymer coating on a silicone substrate \cite{42}, and found that uncoated surfaces attained 80% coverage in just 6h, thus demonstrating that the polymeric coating delayed the colonization. Saldarriaga et al. conducted attachment studies using five species of bacteria incubated for 168h (isolated from implants) on surfaces coated with a crosslinked PEG polymer \cite{54}. They compared the effects of exposure of these surfaces after exposure to bio fluids such as serum, saliva, urine and tears on the bacterial adherence. Their best surface could reduce bacterial attachment by 80% when compared to the glass control surface. A perfectly successful antifouling surface with long term ability to prevent microbial colonization remains a challenge to this day.

The success of bacterial attachment onto surfaces depends mainly on the integrity of EPS as discussed earlier. Damaging the EPS is also an important strategy in preventing bacterial attachment and biofilm removal \cite{34, 51, 55}. This can be achieved by using enzymes such as lysozyme, trypsin, DNase, α-amylase and proteins such as lactoferrin \cite{20, 55-57}. All the enzymes work by cleaving bonds of the polymeric chains of EPS. A detailed introduction on the specific mechanisms involved with various classes of antifouling enzymes will be discussed in Chapter 4. Apart from using anti-adhesive PEG coating, I also used a covalently immobilized protease. Proteases damage the membrane proteins thereby interfering with the initial bacterial attachment. Among the various proteases, subtilisin was chosen as a model enzyme to perform immobilization studies on the PEG and OEG mixed surfaces created by backfilling. The details of enzyme based studies are presented in Chapter 4. Protein adsorption, bacterial attachment and enzyme immobilization involve interactions of biomolecular systems with surfaces. In order to control these processes one has to understand the factors governing the interactions, and this aspect is discussed in the next section.
III. The physical forces involved in interfacial phenomena, physicochemical interactions

The interactions of all biomolecular systems with their environments are driven by the fundamental forces of nature that can be broadly classified into long and short range forces. The main non-covalent interaction based forces relevant to biomolecular systems are electrostatic or (acid-base) interactions, van der Waals forces, hydrogen bonding, dispersive forces and hydration forces [58, 59]. In the case of microscopic systems gravitational, Brownian and hydrodynamic forces also play major roles in their behavior [60]. The forces dominating the behavior of a system are determined by the length scales they influence. For example when considering intermolecular interactions in the case of proteins having nanometer scale dimensions (<10 nm), hydration forces play a significant role, but in the case of microscopic systems, van der Waals and double layer forces dominate [61]. The simple laws of thermodynamics (minimization of energy, maximization of entropy) that apply to chemical systems also govern interfacial biophysical phenomena, but it is difficult to gain a comprehensive theoretical and experimental understanding of these phenomena due to the complex interplay of fundamental forces involved [62]. Apart from the system itself, the solvent properties also change the way a biomolecule interacts with a surface. Some of the main solvent properties relevant to interfacial adsorption processes are listed below:

- **pH** - The state of ionization of functional groups such as carboxyl, amine, phosphate, hydroxyl, thiol and other groups at a given pH depends on their pKa values. Thus the surface charge and also charge of biomolecular systems vary depending on the pH. The efficiency of enzyme immobilization and grafting of polymers is also greatly influenced by pH.

- **Ionic strength** - The Debye length of a charged surface is inversely proportional to the ionic strength of the solution. Whether or not the electrostatic interactions dominate the intermolecular and molecule-surface interactions depends on the Debye length, and hence the ionic strength [63, 64].

- **Temperature** - Changes in temperature can influence stability of weak bonds, solubility, and also alter the kinetic vs thermodynamics competition in chemical reactions, and is also relevant in more complex systems such as protein adsorption [65, 66].

The generally accepted mechanism of biomolecular adsorption starts with attraction to
the surface due to long range attractive forces such as van der Waals forces, which could be acting against or together with electrostatic forces [59, 62]. Reversible attachment is formed if the energetics of interactions is favorable. This reversible attachment paves the way for dispersive hydrophobic interactions based irreversible adsorption. An important implication for immobilization of bioactive proteins such as enzymes on bare substrates are hydrophobic interactions that can cause conformational changes that could render the molecule inactive [67]. The denaturation of immobilized molecules can be minimized by immobilization to hydrogels or hydrophilic polymer layers thereby shielding the hydrophobic patches of substrate. In real environments such as those encountered in biomedical applications or in the food and marine industries is the complex mixture of biomolecules present that result in competitive adsorption phenomena. To begin with the most abundant proteins adsorb to a maximum extent, but as system reaches equilibrium, the proteins with higher affinity tend to replace the loosely adsorbed proteins, this is known as the Vroman effect for blood contacting devices [68, 69]. Once we understand the physicochemical nature of surface-biomolecular interactions, the next step would be to design surfaces that can control adsorption. The forces relevant to adsorption of molecules are also highly relevant to polymeric self assembly processes that are used in numerous applications such as making antifouling surfaces, which is the main theme of this thesis. The forces relevant to polymeric self-assembly process are discussed in the next section.

IV. Self assembly and grafting of non fouling polymeric chains

Surfaces grafted with polymer layers have a wide range of applications such as self cleaning coatings, entanglement of surfaces, antifouling, anti-corrosive, high temperature resistance, cell patterning, tissue engineering, biomolecular immobilization, sensing devices involving protein or DNA microarrays and NEMS/MEMS devices [4, 70-76]. The performance of a surface coated with polymer molecules in a specific application indeed depends on the chemical and physical properties of the polymer used. Apart from the choice of polymer there are several properties of a coating that make it suitable for a specific application, such as the nature of polymer-substrate interactions (covalent, non-covalent), molecular weight of the polymer chains (linear or branched), conformation of the polymer on the surface, and the
pinning or graft density (chains and end groups per unit area). There are numerous types of substrate-head group chemistries that could be used for grafting polymers onto surfaces depending on the availability of reactive chemical groups on the polymer. The dependence of functional stability of SAMs as a function of physicochemical properties of the polymeric molecule was discussed in the ‘Antifouling strategies’ section. The nature of substrate-head group interactions in SAMs is also a very important aspect in regard to the successful application of SAMs. Various kinds of substrates, modification procedures and substrate-polymer interactions are listed below.

a. Substrate
   i. Metal/metal oxide: Au, Ag, Pt, Pd, Ta; oxides of Ti, Fe, Sn, Zr, V, Hf [77-79]
   ii. Organic polymers: PS, PE, PP, PB, PMMA, PDMS, PU, PLA, PVC [80-84]
   iii. Ceramics: alumina, zirconia, silicate glass, calcium phosphate, calcium carbonate [85]

b. Substrate modification
   i. Treatments: Concentrated acid or base, plasma, corona discharge, UV, ozone, flame [84]
   ii. Chemical modification using bifunctional linkers, SAMs with desired tail or end groups, terminating polymerization using required end groups in ‘grafting from’ techniques [80, 81, 84, 86]

c. Substrate-polymer interactions
   i. Covalent: Amide (amine-carboxyl, amine-maleic anhydride, azide- aromatic ester with phosphate group at ortho position), reductive amination (amine-aldehyde), epoxy-amine or thiol or hydroxyl, thioether (thiol-benzyl or acetyl or acetyl halide, click chemistry (alkyne-azide, thiol-ene), siloxane (silanol-hydroxyl), thiol-maleimide/Au/Ag/Pt/Pd, Acid-base (metal oxide-carboxylic or phosphonic acid) [41, 81, 87, 88]
   ii. Electrostatic: Sulfonate on PU, PLL and PEI on oxides [89, 90]
   iii. Biomimetic: Catechol and titanium binding aptamer peptides [91-93]

Surfaces of metal or semiconductor oxide generally have hydroxyl groups that are crucial for a number of chemical modifications and their surface density can be increased by
oxidation procedures such as wet chemical treatments, plasma and UV-ozone [94]. Although organic polymers can be tailored during synthesis to yield surfaces with suitable reactive groups, post synthesis surface modifications are very popular in polymer functionalization. For example, PE surfaces upon UV ozone treatment produce carbon species corresponded to ether, aldehyde, carboxylic carbon [95] and silanol groups are produced on surface of PDMS using the same treatment, as determined by XPS [96]. Plasma based surface modification and polymerization is a versatile tool for biomedical applications [97]. For example plasma polymerization on silica surfaces using allylamine, heptylamine, acetaldehyde produced thin films with amine and aldehyde groups, respectively, which could be further functionalized using reductive amination or carbodiimide chemistry as reported by Griesser et al. [98]. In addition to grafting non-fouling polymers, as demonstrated by Cole et al. [99], the plasma polymerization technique when combined with masked lithography type procedures is capable of creating two dimensional chemical patterns which can help in creating arrays of cells or biomolecules as reported by Thissen et al [100]. Functionalization of surfaces using SAMs is also a popular approach to tailor the surface chemical groups. For example Seifert et al. used biotin terminated alkane thiol SAMs to immobilize streptavidin [101], and in a similar fashion alkyl silanes are mostly used for the functionalization of oxide surfaces [86].

The strength of physical or chemical interaction between the substrate and head group dictates the stability of SAMs in different environments. Covalent based coupling in general is more stable in comparison with electrostatic, hydrophobic, van der Waals interactions. Kingshott et al. demonstrated that PEG chains coupled to covalently immobilized PEI surfaces were more efficient in resisting bacteria when compared with a physisorbed PEI surface [102]. The instability of SAMs is mainly due to desorption due to competitive adsorption or degradation of head group. Thiol based functionalization of noble metals has limited stability in ambient or physiological conditions due the oxidation of thiol to sulfate, which has lower affinity towards noble metals. Cerruti et al. showed that PEG thiol SAMs on Au were prone to thiol oxidation driven desorption upon exposure to air, whereas PEG silane based SAMs on silicon nitride were stable for over two months [103]. Kanta et al. studied the stability of octadecyl phosphonic acid SAMs on titanium surfaces in various solvents and found that the SAMs were not stable in water, ethanol and methanol [104]. Electrostatic
interactions are screened in the presence of high salt concentrations such as situations in marine or physiological environments. Mani et al. showed that there was considerable desorption when SAMs of dodecyl phosphonic acid was exposed to Tris buffered saline at 37 °C while the deodecyl trichloro silane based SAM was stable [105]. The poly(l-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) SAMs are used for the functionalization of a range of negatively charged surfaces based on electrostatic attraction and are very efficient in resisting protein adsorption. These electrostatic attraction based interactions were demonstrated to be unstable under high salt conditions [106]. Blattler et al. showed that covalent based coupling of PLL-g-PEG onto aldehyde groups created by plasma polymerization make the SAM stable at high salt concentrations [106]. A few research groups are also involved in development of stable, universal binding functional groups that are capable of binding to all surfaces. This endeavor gets its inspiration from naturally occurring 3,4-dihydroxyphenylalanine (DOPA) rich mussel adhesive proteins, which are efficient in binding to most surfaces [107]. Catechol functionalized PEG molecules were developed trying to mimic the mussel adhesive protein. Malisova et al. reported detailed studies on surface coverage, serum resistance ability of various types of catechol (different acidity) and nitrodopamine functionalized PEG SAMs on oxide surfaces [108].

SAMs of alkane thiols on gold are one of most widely studied self assembly systems. The typical structure of an alkane thiol SAM is shown in Figure 3a with the labels of the most important parameters of the SAM. The intermolecular van der Waals interactions make SAMs of alkane thiols crystalline and rigid when compared to SAMs of PEG, which are flexible. Though intermolecular van der Waals interactions are absent in PEG monolayers, most authors refer to the PEG functionalization process as molecular self assembly as long as the PEG chain is bound to a moiety that anchors it to the substrate. A molecule capable of self assembly must have a head group to interact with the substrate; a chain group; the end or tail group which mainly controls the interfacial properties such as being helpful in immobilization of biomolecules as shown in Figure 3b. The PEG graft density is the most important factor determining the quantitative ability of PEG surfaces to resist fouling [109], and it has been observed that there is an inverse relationship between the M_W of PEG (also related to Flory radius) and graft density [110] due to the ‘excluded volume effect’ [111]. The graft density of
PEG SAMs determines the conformation of polymeric chains, which could either be in brush, mushroom or pancake conformations [44, 109, 111], with brush being the most effective against fouling [110]. The relationship between the M\text{W} of PEG, Flory radius, and graft density of a SAM are given by Equations 1, 2 and 3:

\[ R_F = a \frac{N^2}{3} \quad \text{Eq (1)}; \quad L = \left( \frac{M}{\rho N_A d} \right)^{\frac{1}{2}} \quad \text{Eq (2)}; \quad S = \frac{1}{L^2} \quad \text{Eqn (3)} \]

\( R_F \) – Flory radius, \( a \) – monomer size in lattice model, \( N \) – number of monomers, \( L \) – interchain distance, \( M \) – molecular weight of PEG chain, \( \rho \) – density, \( d \) – dry thickness, \( N_A \) – Avogadro number, \( S \) – graft density defined as number of chains per unit area.

**Figure 3:** (a) SAM of alkane thiol on a gold substrate with labels showing area per molecule, \( d \) - intermolecular distance, tilt angle \( \theta \). (b) A SAM of PEG with enzyme molecules immobilized on the end groups.

The PEG functionalization techniques can be broadly classified into ‘grafting to’ and ‘grafting from’ approaches (Figure 4). The ‘grafting from’ technique involves growth of polymeric chains from initiators immobilized on a substrate (Figure 4b) and it generally gives higher graft density since the limiting factor is diffusion of monomer molecules onto the reactive ends of growing chains. This is unlike the case of the ‘grafting to’ technique where the limiting factor is the diffusion of the entire polymer molecule to the reactive substrate (Figure 4a) [112]. The limitation of the grafting-to technique of low graft density can be overcome using various strategies such as ‘cloud point’ grafting [41], grafting in homopolymer solutions [113], grafting from polymeric melts [114] and underbrush formation by backfilling
In the ‘cloud point’ grafting technique solvents with high ionic strengths along with high temperatures are used. As discussed in section (1c) under these conditions the Debye length decreases and also the solubility of PEG is greatly diminished in solutions saturated with salts. The high temperature used (higher than LCST of PEG) disrupts the hydrogen bonding between water molecules and PEG, thereby further decreasing the solubility [116]. The presence of ions has been shown to alter the solubility, also lowering the critical solution temperature [117]. The extent of decrease in solubility of a non-electrolyte due to the presence of an ion is directly related to its polarizability. Anions in general have higher polarizability and thus are more important in applications such as ‘cloud point grafting’ where the solubility of polymer is decreased [117]. Under these conditions the PEG chains exist in a collapsed state rather than an extended conformation. Grafting under these conditions thus results in higher graft density. The backfilling based technique unlike the other three strategies that depend on minimization of excluded volume interactions, is a simple method wherein the interchain spaces present in layers of higher \( M_w \) PEG SAMs are backfilled with shorter OEG chains that can diffuse to the surface. In this thesis two strategies were used for maximization of the graft density; firstly backfilling using OEG which is discussed in Chapter 3 and secondly grafting under reduced solubility conditions using supercritical carbon dioxide, as discussed in chapter 5.

**Figure 4:** (a) Schematic of the ‘grafting-to’ polymer grafting technique and, (b) Various steps involved in the grafting-from technique.
CHAPTER 2: EXPERIMENTAL TECHNIQUES

I. Introduction to surface characterization in biointerfaces

Understanding the behavior of surfaces in biological environments requires thorough physical and chemical characterization to correlate the observed biointerfacial events to the properties of the material surface [118]. Some of the main properties of surface coatings that influence biointerface phenomena are film thickness or coverage, conformation or orientation of the constituents of the film, chemical composition, wettability or surface energy, charge, optical properties, refractive index, surface topography and mechanical stiffness [118-120]. The quantification of the content of chemical groups on surfaces can be done directly or by derivatization using suitable probe molecules [118-120]. Once the material surface has been characterized, the next challenge would be to gain knowledge about the changes that occur at the interface when the surface is exposed to a biological environment. Upon exposure to a biological environment, the physical and chemical properties of the surface may change due to a lack of stability of the interface; apart from the intrinsic changes at the interface, the interface itself might get buried under a layer of adsorbing molecules, thereby creating a new interface which then decides the fate of material surface in a given environment [12]. Thus, the knowledge of quantitative and qualitative aspects of adsorption events occurring at material interfaces exposed to complex biological environments is absolutely essential to comprehend the behavior of materials in biological environments that can be used in order to optimize the surface for improved performance [118-120]. Real-time characterization of various changes occurring at an interface would be ideal, but many surface characterization techniques are unable to analyze samples at ambient or under aqueous conditions. Table 1 gives an overview of some of the most widely used surface characterization techniques used in biointerface science. The choice of technique is based on its ability to provide the required information, optimal resolution and required sensitivity.
Table 1. Overview of commonly used surface characterization techniques [1, 2, 118, 120, 121]

<table>
<thead>
<tr>
<th>Technique</th>
<th>Vertical resolution</th>
<th>Lateral resolution</th>
<th>Sensitivity</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>Surface</td>
<td>&lt; 1nm</td>
<td>high</td>
<td>Mainly visualization; ambient conditions, liquid conditions</td>
</tr>
<tr>
<td>Contact angle</td>
<td>3-20Å</td>
<td>1mm</td>
<td>Low or high</td>
<td>Swelling, extraction by solvent</td>
</tr>
<tr>
<td>STM</td>
<td>5Å</td>
<td>1Å</td>
<td>atoms</td>
<td>Only conducting samples, ambient conditions possible, no liquid studies</td>
</tr>
<tr>
<td>SIMS, ISS</td>
<td>10Å-1μm</td>
<td>500Å</td>
<td>Very high</td>
<td>Operates in vacuum, Sample damage (except static mode), semi quantitative</td>
</tr>
<tr>
<td>Ellipsometry</td>
<td>≃1nm</td>
<td>1mm</td>
<td>High</td>
<td>Operates in ambient condition and liquid. Affected by surface roughness, scratches</td>
</tr>
<tr>
<td>SEM</td>
<td>15Å</td>
<td>40 Å</td>
<td>High not quantitative</td>
<td>Operates in vacuum. Sample damage, sample preparation artifacts</td>
</tr>
<tr>
<td>XPS/ESCA</td>
<td>10-250 Å</td>
<td>10-150μm</td>
<td>0.1 atom%</td>
<td>Operates in vacuum, capable of destructive and non destructive depth profiling</td>
</tr>
<tr>
<td>AES</td>
<td>50-100 Å</td>
<td>100 Å</td>
<td>0.1 atom%</td>
<td>Severe sample damage, quantitation</td>
</tr>
<tr>
<td>Vibrational spectroscopies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HREELS</td>
<td>10 Å</td>
<td>1μm</td>
<td>1mol%</td>
<td>Water signal subtraction</td>
</tr>
<tr>
<td>IRAS</td>
<td>100Å</td>
<td>1μm</td>
<td>High</td>
<td>Limited for depths &lt;1μm</td>
</tr>
<tr>
<td>ATR-IR</td>
<td>1-5μm</td>
<td>1μm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raman</td>
<td>&gt;1μm</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The surface chemistry of materials can be determined using XPS, SIMS, ATR, HREELS, while information about the distribution of groups require imaging techniques. The knowledge of molecular orientation can be obtained from polarized IR, HREELS, NEXAFS,
and imaging techniques like SEM, AFM and STM provide information on the texture of surface. The modulus information of surface can be obtained from AFM and the surface energy deduced from contact angle measurements. There are several ways of classifying the above mentioned techniques. One of the most important classifications is based on whether a technique is capable of performing real-time, *in situ* studies or not. Among the above mentioned techniques, ellipsometry, SPR, AFM and ATR-IR are capable of *in situ* studies under physiological conditions involving biomolecular adsorption processes [122, 123]. Optical waveguide based techniques such as OWLS have also become very popular in biointerfacial studies due to their high sensitivity and good resolution (range 1nm-5μm, resolution 1Å)[124]. In this thesis QCM was used to perform real-time serum adsorption studies on PEG coated surfaces. Also XPS was used to estimate the dry thickness of PEG layer, adsorbed or immobilized protein films. The thickness estimation was also done using ellipsometry to cross check the XPS based measurements. The thickness of thin films can be estimated using other sensitive techniques such as stylus profilometry which has a resolution of 5Å; interferometry which has a resolution of 30Å; using surface plasmon resonance (SPR), resolution <1nm; [125-127]. In the following section of this chapter, the working principles behind the techniques used in this thesis are presented.

II. **X-ray photoelectron spectroscopy (XPS)**

XPS is a quantitative surface spectroscopic technique, providing information about the presence and quantity of the elements (except H and He), the chemical states of each of the elements, and the lateral and vertical distributions. The photoelectric effect was discovered by H.R. Hertz in 1887, and the theoretical basis was provided by Einstein in 1905. Though several scientists studied the photoelectric effect, it was Kai Seigbahn who first demonstrated that XPS can be used for quantitative chemical state analysis and coined the term ‘ESCA’ (electron spectroscopy for chemical analysis). This technique works by analysing the photoelectrons that are ejected from the core level atomic shells of surface atoms during exposure to incident x-rays as shown in Figure 1. The surface sensitivity of this technique is due to the low attenuation lengths of photoelectrons, thus only the top few atomic layers of a
surface contribute to the signal intensity. The relation between the incident photon energy and the photoelectron is given by the photoelectric law given in Equation 4 [1, 2].

Photoelectric law in XPS:

\[ E_{KE} = h\nu - E_{BE} - \Phi_{Sp} \]  

Where, \( E_{KE} \) – kinetic energy of photoelectron, \( h\nu \)– energy of incident photon, \( E_{BE} \) – binding energy of core electron, \( \Phi_{Sp} \) – Work function of spectrometer.

**Figure 1.** The photoionization process

**Figure 2:** Block diagram of components of a XP spectrometer [1, 2]

A x-ray photoelectron spectrometer typically consists of several components as shown in Figure 5 [1]. The x-ray source consists of either a thin film of Al or Mg on a water cooled copper anode, which is bombarded with electrons from a filament, producing x-ray lines with 1486.7 eV or 1254 eV, respectively, and a background due to Bremstrahlung radiation. Monochromatic x-ray sources use a diffracting crystal to remove the background. For
example, quartz crystals are used to obtain the monochromatic Al$_{\alpha}$ line. In the case of conducting samples, the positive charge developed during the photoemission process gets discharged due to its electrical contact with the holder. The neutralization of positive charge developed on an insulating material would require an external source of low energy electrons, which is provided by a flood gun. An alternate solution uses thin grid of conducting material deposited over the insulating surface. The ejected photoelectrons enter the analyser where, depending on the mode of operation, they are either retarded to constant pass energy (fixed analyser transmission or constant resolution mode), or analysed with varying detector pass energies (fixed retard ratio mode). The most commonly used analyser is a hemispherical analyser (HAS) [1]. The photoelectrons are counted by an electron detector that is generally a channel electron multiplier (CEM) producing a cascade of electrons for each photoelectron generated from the sample.

Generally two kinds of spectra are recorded, namely a wide energy scan or a survey spectrum and high resolution spectra, which are shown in Figure 3. A survey spectrum has several primary features, such as the background with step like appearance due to inelastically scattered electrons, which are also the primary source of noise. Auger peaks also appear, which can be differentiated from XPS peaks by altering the energy of incident x-rays. Non-monochromated sources produce satellite peaks, and x-ray sources with eroded anode films can produce ghost peaks from the underlying copper substrate. Photoemission peaks from any orbital other than S orbitals always result in a doublet due to LS coupling; the relative ratio of the doublet depends on the orbital, and difference in their binding energies depends on the splitting constant. Aromatic molecules produce shake-up satellite peaks; metallic surfaces produce plasmon loss peaks and also peak asymmetry is commonly observed on the higher binding energy side of peaks due to loss of photoejected electron energy. X-ray fluorescence is a process that competes with photoelectric emission. The measured intensities from different elements obtained from a survey spectrum can be used to calculate the relative atomic percentages of elements from the Equation 5 [1].

\[
C_A = \sum \frac{(I_A/S_A)}{(I_n/S_n)} \quad \text{Eq (5)}
\]

Where $C_A$ is the relative atomic concentration, $I_A$ is intensity due to element A and $I_n$ is
the sum total of intensities from all the elements present on the surface, S is the relative sensitivity factor.

**Figure 3:** Example of a survey spectrum and a high resolution C 1s spectrum recorded from a SAM of COOH-PEG thiol on gold [data from Chapter 3].

\[ d = 3 \lambda_{AL} \sin \theta \]  \hspace{1cm} \text{Eqn (6)}

Where, \( d \) is escape depth, \( \lambda_{AL} \) is PE attenuation length and \( \theta \) is take off angle wrt the normal to the surface.

The intensity emanating from atoms at depth \( Z \) is given by:

\[ I_Z = I_0 e^{-\frac{Z}{\lambda \sin \theta}} \]  \hspace{1cm} \text{Eqn (7)}

Where \( I_Z \) is intensity at \( Z \), \( I_0 \) intensity from surface atoms of bulk material, \( \lambda \) and \( \theta \) are the same as in the Equation (6).

**Figure 4.** Schematic of a substrate being analysed in angle resolved XPS (modified from [1]).

The thickness of overlayer on substrates can be calculated by knowing the extent of attenuation of the substrate signal using Equation (7), which can be integrated to give Equation (8) [1, 2].
where $Z$ - overlayer thickness, $\lambda$ – attenuation length, $\theta$ is the take-off angle, $I$ is the substrate signal with thin film coating, $I_0$ bare substrate intensity.

Alternately the overlayer thickness can be determined using signal from any of the elements present in the overlayer ($I$), if the elemental signal from an infinitely thick overlayer ($I_0$) is known, given by Equation (9)

$$-z = \lambda \cos \theta \ln \left( \frac{I}{I_0} \right) \quad \text{Eqn (9)}$$

High resolution spectra allow the quantification of various chemical species of an element with different oxidation states due to variations in chemical bonding environment surrounding each atom, which can be explained by charge potential model [1]. An example of a high resolution C 1s spectrum of a SAM of carboxyl capped PEG thiol is shown in Figure 3. The surface sensitivity of XPS is determined by the inelastic mean free path (IMFP) of the photoelectron (PE) or attenuation length ($\lambda_{AL}$), where the analysis depth is approximately three times the escape depth, which is given by Equation (6) [1, 2]. By changing the take off angle with respect to the analyser, the sampling depth can be varied thereby enabling compositional depth profiles. Alternatively depth profiling can be achieved using high energy x ray sources or using layer by layer sputtering.

In this thesis the thickness of grafted PEG and/or OEG layers on gold substrates was calculated using Equation (8), using the gold signal. There is always a possibility that a fraction of PEG molecules are physisorbed instead of being covalently bound. Castner et. al. reported a way to estimate the proportion of physisorbed alkane thiol molecules in SAM on gold using high resolution S 2p spectra [128]. The chemisorbed thiolated species on gold can be distinguished from the physisorbed molecules based on the difference in core electron binding energies of covalently bound and unbound sulphur atoms. An example of a peak-fitted high resolution S2p spectrum is shown in Figure 5.
II. Quartz crystal microbalance (QCM)

QCM is a sensitive surface analytical technique capable of giving quantitative information on interfacial processes resulting from changes in mass loading on a quartz crystal. The theoretical and experimental basis of QCM was proposed and demonstrated in 1959 by Sauerbrey [129]. The use of QCM in the field of biointerfaces started in the 1980s and has evolved into a valuable technique in understanding interfacial phenomena such as protein adsorption, and is particularly useful for studying fouling processes. A typical QCM sensor is shown in Figure 9a, consisting of a quartz crystal sandwiched between an active and a counter electrode. The piezoelectric quartz crystal is actuated by applying a potential across the electrodes. The frequency of vibration is sensitive to the mass of the interfacial overlayer on the electrodes (Figure 6b). It enables quantification of changes in mass due to adsorption or desorption occurring on the crystal using the Sauerbrey equation given in Equation (9a) [43]. This equation is an approximation and assumes that the viscoelastic
properties of the interface remain unchanged while the process is being monitored. The use of the Sauerbrey equation for systems with varying viscoelastic properties would result in underestimations of mass. Performing studies using multiple frequencies and knowledge of dissipation given by Equation 9b enables the accurate estimation of mass, and viscoelastic properties of films using the Voigt model [130, 131].

\[
\Delta m = -\frac{C}{n} \Delta f \quad \text{Eqn (9a)}
\]

\[
D = \frac{E_{\text{Dissipated}}}{2\pi E_{\text{Stored}}} \quad \text{Eqn (9b)}
\]

Where, \( \Delta m \) – change in overlayer mass, \( C = 17.7 \text{ ng Hz}^{-1} \text{ cm}^{-2} \) for 5 MHz quartz crystal, \( n \) – overtone number, \( \Delta f \) – change in frequency, \( D \) – dissipation, \( E_{\text{Dissipated}} \) – energy lost, \( E_{\text{Stored}} \) – energy stored.

QCM has been used in a number studies in biomedical sciences, such as biorecognition, cell-surface interaction studies, lipid bilayer formation and fouling processes [91, 131, 132]. QCM is a sensitive and versatile tool to study the qualitative and quantitative aspects of protein adsorption process on a variety of antifouling surfaces such as oligo(ethylene glycol) [133, 134] and poly(ethylene glycol) [132, 135] SAMS. In this thesis, QCM was used to ascertain the ability of PEG functionalized surfaces to resist protein adsorption. Surfaces were tested with solutions of single proteins and complex bovine serum. An example of a QCM plot involving fetal bovine serum (FBS) adsorption on various substrates is given in Figure 7. The advantage of QCM is that it allows the studies to be conducted without the need for any probe labels, at variable temperature, ambient pressure, and importantly in aqueous conditions, which more closely mimic ‘real’ biological environments. The results of protein adsorption studies using QCM are discussed in Chapters 3 and 5.
**Figure 6.** (a) Shows the two sides of typical QCM crystal with the various electrodes labeled. (b) Schematic of actuation of quartz layer sandwiched between electrodes [136].

**Figure 7.** An example of a QCM graph showing the change in frequency during the adsorption of 10% FBS on SAMs of oligo(ethylene glycol) (OEG), poly(ethylene glycol) (PEG), alkane (R) and gold, 750 μl of 10% FBS solution, flow rate 100 μl/min, 37°C, followed by PBS rinsing. The time points of serum exposure and PBS rinse are indicated with arrows [data from Chapter 3].
III. Contact angle based surface energy measurements

Contact angle measurements have a very high surface sensitivity (<1nm) [137] and allow the determination of the surface energy of materials [138]. In 1988 Van Oss and R.J. Good provided the theoretical background to correlate the contact angle of liquids on surfaces with the surface energy components. The components of surface tension can be calculated by measuring contact angles of three different liquids and solving the three simultaneous equations given in Equation (10) [138]. The surface tension components of three liquids used in this thesis to measure the surface energy components of various surfaces are given in Table 2. Surface hydrophobicity is determined by the $\gamma_s^d$ value; the lower the value, the more hydrophobic a surface is. The $\gamma_s^-$ values give a quantitative idea about the electron donor groups; the greater the value of $\gamma_s^-$, the higher the number of electron donor groups on a surface; and the $\gamma_s^+$ values represent the number of electron acceptor groups. Surface energy measurements were used to ascertain the homogeneity of mixed monolayers of PEG and OEG created by backfilling. There is a possibility that backfilling might result in islands of PEG and OEG similar to phase separated type arrangements [139, 140]. Since the surface wettability of PEG and OEG would be very similar, we used octane thiol for backfilling experiments instead of OEG in order to follow the mechanism of addition. Alkane molecule being more hydrophobic than PEG molecule, it would be possible to check for the presence of islands if at all formed during backfilling by octane thiol using surface energy studies. The results of these studies are presented and discussed in Chapter 3.

**Table 2**: Lifshitz van der Waals component ($\gamma_s^d$), electron acceptor component ($\gamma_s^+$), electron donor component ($\gamma_s^-$) of water, formamide and $\alpha$-bromonapthalene [138].

<table>
<thead>
<tr>
<th>Liquid</th>
<th>$\gamma_s^d$ mJ/m$^2$</th>
<th>$\gamma_s^+$ mJ/m$^2$</th>
<th>$\gamma_s^-$ mJ/m$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>21.8</td>
<td>25.5</td>
<td>25.5</td>
</tr>
<tr>
<td>Formamide</td>
<td>39</td>
<td>2.28</td>
<td>39.6</td>
</tr>
<tr>
<td>$\alpha$-Bromonapthalene</td>
<td>44</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
\[ (1 + \cos \theta) \gamma_L = \frac{1}{2} \left[ (\gamma_S^d \gamma_L^d)^{\frac{1}{2}} + (\gamma_S^+ \gamma_L^+)^{\frac{1}{2}} + (\gamma_S^- \gamma_L^-)^{\frac{1}{2}} \right] \quad \text{Eqn (10)} \]

\( \theta \) – Measured contact angle. \( \gamma_L, \gamma_L^d, \gamma_L^+ \) and \( \gamma_L^- \) represent the total surface tension, the Lifshitz van der Waals component, the electron acceptor component, and the electron donor component of liquid, respectively. \( \gamma_S^d, \gamma_S^+ \) and \( \gamma_S^- \) represent the Lifshitz van der Waals component, the electron acceptor component, and the electron donor component of a surface, respectively.

IV. Atomic force microscopy (AFM)

AFM is a scanning probe microscopy technique, which has the advantage that the samples analyzed do not need to be conducting, and the analysis does not have to occur in vacuum. Biological specimens can thus be analyzed under ambient conditions or even in liquid [141]. The first AFM was built by Quate and Gerber in 1986. The basic working setup of an AFM is given in Figure 8a. It consists of a sharp tip mounted on an optical lever facing the sample while a laser beam is reflected from the opposite side of the tip. The reflected laser beam is incident on a position sensitive photodiode (PSPD), thereby enabling the conversion of tip-sample interactions into an electrical signal, which is used to construct topographic images of the area being scanned. Apart from imaging, AFM can record force-distance curves (Figure 8b). Using these curves, it is possible to study forces in the pico Newton range that are involved in molecular interactions with surfaces. These studies can give valuable information such as graft density [142], molecular conformation and frictional properties [143] of interfaces grafted with polymers [141], helping to understand complex interfacial phenomena relevant to fouling processes. The traditional way of acquiring force-distance curves involves an approach-retraction procedure from a large number of positions on a sample followed by analysis all the recorded curves to provide statistically meaningful results. In this thesis, the topography and elastic modulus properties of PEG functionalized surfaces were mapped using Quantitative Nano-Mechanical Mapping (QNM)-AFM under Peakforce Tapping mode conditions. The force-distance curves were fitted by the Derjaguin-Müller-Toporov (DMT) model [144, 145]. QNM-AFM enables one to map mechanical properties of
samples quantitatively, while simultaneously capturing topographic images at resolutions as good as traditional tapping mode AFM [146, 147]. The modulus values were used to confirm the absence of phase separated islands in mixed SAMs, and the results of these studies are discussed in Chapter 3.

![AFM Diagram](image)

**Figure 8.** (a) A typical experimental setup used in AFM. (b) A typical force-distance curve consisting of approach and retraction curves.

V. **Ellipsometry**

Ellipsometry is a surface sensitive technique that determines the thickness of surface layers ranging from angstroms to tens of micrometers. It works by measuring the complex refractive index given by Equation 10a or alternatively the complex dielectric function of any material given by Equation (10b) [148, 149]. Drude in 1888 was the first to realize the sensitivity of ellipsometry towards measuring the thickness of monolayer films. This was followed by development of a matrix formalism by Abeles in 1947, who applied ellipsometry to multilayered thin films. A typical ellipsometer setup is shown in Figure 9. The probe is a linearly polarized laser beam with field components $E_p$ and $E_s$ in the directions parallel and perpendicular to the plane of incidence of the light. It works by analyzing the extent of attenuation and phase shift in the s- and p-components of incident linearly polarized light that

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28 | P a g e
becomes elliptically polarized upon reflection. Ellipsometry was used in this thesis as a complementary tool to measure overlayer thicknesses of PEG SAMs on gold. The calculations were performed similar to Unsworth et. al. [150]. The PEG modified surfaces were analyzed using a three-layer air–PEO–gold model.

\[ \hat{n} = n + i k \quad \text{Eqn (10a)} \quad ; \quad \varepsilon = \varepsilon_1 + i \varepsilon_2 \quad \text{Eqn (10b)} \]

Where, \( \hat{n} \) – complex refractive index, \( n \) – refractive index, \( k \) – extinction coefficient, \( \varepsilon_1 \) – real dielectric constant, \( \varepsilon_2 \) – complex dielectric constant.

**Figure 9.** A typical experimental setup used in ellipsometry showing a beam of plane polarized light becoming elliptically polarized after reflection accompanied by attenuation and phase shift shown on the ellipse [56][149].
CHAPTER 3
Mixed poly(ethylene glycol) (PEG) and oligo(ethylene glycol) (OEG) layers on gold as non-fouling surfaces

I. INTRODUCTION

The relevance of non-fouling coatings in a variety industrially and clinically applications was discussed in Chapter 1 [3, 4]. Biofilm formation is proposed to be facilitated by a conditioning layer, which is followed by reversible physiochemical attachment of microbes [151]. Among the various strategies used in making non-fouling coatings, preventing initial microbial attachment is a popular approach that generally involves the use of a hydrophilic polymeric coating. Polyethylene glycol (PEG) is widely used to functionalize surfaces in order to render them non-fouling. The osmotic and elastic properties of PEG along with its neutral charge and non-toxic nature make it an effective and safe choice to minimize fouling in a number of biologically relevant applications [45]. The ability for a given PEG functionalized surface to resist fouling is thought to be mainly dependent on the graft density and molecular weight (MW) of PEG used, both of which are key properties that decide the conformation of PEG chains on surfaces [109, 110, 152]. For example, in the case of high or low graft density, PEG chains are arranged in either a ‘brush’ or ‘mushroom’ conformation respectively [150]. Self-assembled monolayers (SAMs) of PEG chains are known to resist non specific adsorption best when present in the brush conformation at high graft densities [109]. Studies have shown that covalently bound PEG coatings also provide superior antifouling properties, because they cannot be easily displaced by conditioning layer molecules [102]. PEG functionalization techniques can be broadly classified into ‘grafting to’ and ‘grafting from’ approaches. The advantage of ‘grafting from’ over ‘grafting to’ technique was discussed in chapter 1. Strategies such as ‘cloud point’ grafting [41], grafting in homopolymer solutions [113], grafting from polymeric melts [114] and underbrush formation by backfilling with shorter molecules [115] could be used to increase the graft density of polymer layers grafted using ‘grafting to’ technique. The backfilling approach, unlike the other three strategies that depend on minimization of excluded volume interactions, is a simple method wherein the interchain
spaces present in layers of higher $M_W$ PEG chains are filled with shorter PEG chains that can diffuse to the surface. Reducing non-specific adsorption by backfilling strategy is well known strategy. For instance Uchida et al. showed that backfilling self assembled layer of 5 kDa PEG thiol with shorter PEG thiol ($M_W$ 2 kDa) improved the ability of 5 kDa PEG SAM to resist non specific protein adsorption [115, 153]. Lee et al. studied the effects of backfilling a self assembled layer of thiol functionalized single strand DNA on gold with OEG capped-11-mercapto-1-undecanol molecules and found that backfilling improved the specific binding based hybridization of complementary DNA strands [154]. Also Lee et al. observed that prolonged exposure of SAM of thiol functionalized single strand DNA to OEG alkyl thiol molecules resulted in the replacement of DNA strands through desorption. The aspect of desorption was not dealt with in reports by Uchida et al., whose backfilling studies are highly relevant to the studies reported in this chapter. Also there is a possibility of islands of short PEG thiol and long PEG thiol SAMs instead of homogeneous incorporation of short PEG thiol molecules into the vacancies found in the SAM of long chain PEG thiol. The arrangement of protein repelling molecules in a mixed layer formed by backfilling greatly influences the antifouling ability of mixed SAMs. For instance if islands are formed, the protein repelling ability of long chain islands would not have improved while the islands of short chain molecules would have higher resistance towards protein adsorption. Mixed monolayers can consist of either islands or homogeneous mixed layer. Examples of both types of mixed monolayer formed by backfilling or co-adsorption can found in literature. For instance Herne et al. obtained backfilling by exposing SAMs of thiolated DNA strands to mercaptohexanol, and found that physically adsorbed DNA strands get replaced upon backfilling [155]. Brunner et al. observed that phase separated islands were formed by replacement when SAM of poly($p$-phenylene) was exposed to octadecane thiol [156]. Tosatti et al. studied the SAMs formed by co-adsorption of dodecyl phosphate and hydroxyl dodecyl phosphate on titanium oxide [157]. They showed that homogenous mixed monolayers with no phase separation, thus enabling the systematic variation of wettability properties of a surface by changing the mole fractions of two compounds in solution. Bozzini et al. performed co-adsorption experiments using PEG functionalized alkane phosphate and hydroxy alkane phosphate, and demonstrated that the graft density of PEG chains could be varied systematically by varying
the relative mole fractions in solution, also the fouling properties of surfaces changed accordingly [158]. Amstad et al. stabilized iron oxide nanoparticles using a mixed monolayer formed by co-adsorption hydroxy dopamine-PEG and biotin tagged hydroxy dopamine-PEG, and demonstrated that the surface density of biotin could be varied by changing the relative mole fraction [159]. Apart from the above mentioned studies, one can find reports on alkane thiol co-adsorption and backfilling experiments, which have been shown to produce phase separated islands [139, 140, 160].

The mechanism of formation of mixed layers by backfilling is not yet fully understood for SAMs of PEG thiol exposed to shorter thiol molecules, similar to the study by Uchida et al. [115, 153]. The possibility of the formation of domains exists (Figure 1a), where the mechanism of addition is similar to that of the replacement phenomena observed for short alkane thiol SAMs when they are exposed to longer alkane thiol molecules [139]. Since alkane thiol self-assembly is driven by intermolecular van der Waals interactions and are kinetically more reactive than high MW PEG thiols, the aim this study was to thoroughly explore whether phase separated domains are formed (Figure 1a), and also the extent of PEG thiol desorption. The structures of the thiolated PEG, OEG and alkane molecules used are presented in Figure 1c. Although SAMs of OEG have been shown to be very efficient at resisting protein adsorption [46] for short durations, it has been identified that applications involving immobilization of bioactive molecules such as enzymes would benefit from longer PEG chains due to higher mobility and substrate accessibility during biorecognition [161]. Thus it is expected that the combination of PEG-COOH\textsubscript{SAM} backfilled with OEG\textsubscript{3} will be an ideal support for immobilizing bioactive molecules. The use of antifouling enzyme as one of the strategies to prevent biofilm formation was discussed in chapter 1. Thus the backfilled PEG-COOH\textsubscript{SAM} would be ideal support for immobilization of antifouling enzymes such as proteases and the results of this study are presented in chapter 4.
Figure 1. The schematic diagrams of backfilled PEG SAMs in island type (a) and underbrush type arrangements (b). The chemical formula and assigned abbreviations for various molecules used in self assembly and backfilling experiments (c).

The following notations for SAM of PEG and for the backfilled surfaces are used:

- PEG-COOH\textsubscript{SAM}, OEG\textsubscript{3-SAM}, and R\textsubscript{SAM} correspond to SAMs formed with PEG-COOH, OEG\textsubscript{3}, and R molecules.
- PEG-COOH\textsubscript{SAM}+OEG\textsubscript{3}: PEG-COOH\textsubscript{SAM} backfilled with OEG\textsubscript{3}
- PEG-COOH\textsubscript{SAM}+R: PEG-COOH\textsubscript{SAM} backfilled with R

II. MATERIALS AND METHODS

A. Substrate preparation and functionalization

Chemicals α-carboxyl-ω-thiol poly(ethylene glycol) (PEG-COOH, 99% purity, Mw-2000Da) was purchased from Laysan Bio Inc. (Alabama, USA), and hydroxy-terminated
tri(ethylene glycol) undecanethiol (OEG₃, 99% purity) was purchased from Assemblon (Washington, USA). Fetal bovine serum (FBS), octanethiol (R) (99% purity), SYBR Green II, phosphate buffered saline (PBS) buffer tablets, 25% ammonium hydroxide (NH₄OH), 30% hydrogen peroxide (H₂O₂) and absolute ethanol were purchased from Sigma Aldrich (Aarhus, Denmark). Ultrapure MilliQ (MQ) water with resistivity of 18.2 MΩ was used for making buffers and aqueous ethanolic solutions.

**B. Substrate preparation and functionalization**

Gold substrates were prepared by sputtering 50 nm gold layer onto a 3 nm titanium attachment layer on silicon wafers. Sputtering was done using a RF sputtering system with Ti and Au targets of 10 cm diameter (2.54 W cm⁻²) inside a standard chamber maintained at an Ar pressure of 2 × 10⁻³ mbar. The substrate-target separation distance was 7 cm, while the deposition rates for Ti and Au were 0.4 nm s⁻¹ and 1 nm s⁻¹, respectively. The gold surfaces were cleaned by UV/ozone treatment for 30 minutes, followed by treatment with basic piranha solution (H₂O: NH₄OH: H₂O₂ in ratio 4:1:1) at 70˚C-80˚C for 5 minutes and then rinsed with MQ water. (CAUTION: Piranha solution reacts violently upon contact with organic solutions). Cleaned gold slides were immersed for 3 h at room temperature in 0.2 mM PEG-COOH solution (75% ethanol), followed by rinsing with MQ water to remove any physically adsorbed molecules. Backfilling involved immersion of PEG-COOHₐₕ for 3 h at room temperature in 1mM absolute ethanolic solution of OEG₃ or R, followed by rinsing with ethanol and drying with a jet of nitrogen. The SAMs of OEG₃ and R for control experiments were made by immersing cleaned gold slides in 1mM ethanolic solution of OEG₃ or R for a period of 3 h followed by rinse with ethanol and drying with nitrogen gas.

**C. X-ray photoelectron spectroscopy**

XPS spectra were recorded using a Kratos Axis Ultra DLD instrument (Kratos Ltd, Telford, UK) equipped with a monochromated aluminum source (AlKα 1486 eV) operating at a power of 150 W (15 kV and 10 mA) with pass energies of 80 eV and 160 eV for high resolution and survey spectra, respectively. A hybrid lens mode was employed during analysis (electrostatic and magnetic). The XPS spectra were measured at three areas on each sample and the take-
off angle for all measurements was 0°. The measured binding energy positions were charge corrected with reference to 285.0 eV, corresponding to the C-C/C-H species. Quantification and curve fitting was conducted using CasaXPS software. A linear background with a Gaussian to Lorentzian ratio of 30 was used to fit all spectra. The S2p high resolution spectra were peak fit into two doublets corresponding to bound and unbound thiol each with full width half maximum of 1.2 eV. The doublet corresponding to photoemission from 2p orbital has two peaks in the ratio 1:2 which can be assigned to 2p_{1/2} and 2p_{3/2} respectively. The high resolution S2p spectra with peak fitting of various surfaces analyzed using XPS are shown in Figure 2. The lower binding energy peak of the doublet with its 2p_{3/2} at 161.9 eV was assigned to bound thiol while the higher binding energy doublet with its 2p_{3/2} peak at 163.5 eV corresponded to unbound thiol as described by Castner et al. [128]. Overlayer thickness and graft density were calculated as described in Chapter 2.

D. Contact angle measurements

Static contact angles were measured on all surfaces using water, formamide and α-bromonaphthalene. All images of liquid drops on surfaces were recorded using a KRUSS DSA100 (KRUSS GmbH, Hamburg, Germany), followed by drop shape analysis using ImageJ software. Reported contact angles are the average of at least 6 measurements at different surface positions. The values of \( \gamma_{L^d} \), \( \gamma_{L^+} \), \( \gamma_{L^-} \) for the three liquids used are given in Table 1 in Chapter 2. The interfacial Lifshitz van der Waals (\( \gamma_S^d \)) and polar components (\( \gamma_S^+ \), \( \gamma_S^- \)) of surface tension of a surface can be determined by measuring contact angles with three different liquids followed by solving the corresponding equations whose general form is given by equation 10 in Chapter 2 [138].

E. Atomic force microscopy

All of the AFM images were recorded using a commercial Nanoscope VIII MultiMode SPM system (Bruker AXS, Santa Barbara, CA) in MQ water. Topography images were recorded using Quantitative Nano-Mechanical Mapping (QNM) under Peakforce Tapping mode [147]. Ultrasharp silicon nitride cantilevers (triangular, Mpp-12120-10, Bruker AXS) were used with a typical resonance frequency of 150 KHz in air, a spring constant of 5 N/m
and a normal tip radius of 8 nm. All AFM images were recorded with 512 × 512 pixels resolution per image, and they were flattened and analyzed with Scanning Probe Image Processor software (SPIP™, Image Metrology ApS, version 5.1.3, Lyngby, Denmark).

F. Quartz crystal microbalance with dissipation

QCM-D studies were performed using a Q-Sense E4 system (Gothenburg, Sweden). The PEG-COOH functionalization followed by backfilling with OEG₃ or R was performed on QSX 301 crystals (Q-Sense) coated with 100 nm gold which had approximate resonance frequency of 5 MHz. Serum adsorption experiments were performed in triplicate. Functionalized gold coated crystal surfaces were equilibrated at 37 °C in PBS (100 mM pH 7.4) prior to exposure to serum solution. All serum adsorptions studies were carried out using 10% fetal bovine serum (FBS) at 37 °C. Once a stable baseline was attained, the surfaces were exposed to 750 μL of protein solution delivered at a flow rate of 100 μL/minute followed by rinsing with PBS buffer. The shift in resonance frequency corresponding to the 7th overtone was used to monitor the adsorption. After the QCM crystals were exposed to serum they were rinsed with MQ water to remove any salts before drying under a jet of nitrogen for XPS analysis.

G. Bacterial attachment assay

A Staphylococcus aureus (ATCC 12598) starter culture was inoculated from agar plates and grown in 3 ml of 1% tryptic soy broth (TSB) medium in 50 ml conical bottom tube by incubating overnight in a shaker at 37°C. An inoculum prepared by adding 1 ml of starter culture to 100 ml 1% TSB medium was incubated in a shaker at 37°C until cultures were in the late-exponential growth phase (OD₆₀₀ = 1.0). Cells were then harvested by centrifugation (5 minutes at 3000 x g), washed twice and resuspended in PBS (pH 7.4) and diluted to obtain an OD₆₀₀ of 0.5. Surfaces were incubated with the bacterial suspensions in 24 well plates for 5 h at 37°C and gently washed three times with sterile PBS to remove non-adherent cells. The adherent cells on the surfaces were stained with ~10 μl 20x SYBR Green II RNA (2 μl ml⁻¹ of 10,000x SYBR Green II stock), covered with glass cover slips and sealed with nail polish to avoid evaporation. Slides were stored in the dark at 4 °C until analyzed. Adherent cells were counted by fluorescence microscopy using a Zeiss Axiovert 200M epifluorescence
microscope (Carl Zeiss GmbH, Jena, Germany) equipped with Zeiss filter set 10 and 63x oil immersion objective. Cells were counted manually in 109 µm² grids on triplicates of sample on a total of fifteen random positions on each of the surface type. A student t-test was used to assess the significance of difference in bacterial attachment count on different surfaces.

III. RESULTS AND DISCUSSION

A. Extent of addition and surface coverage in backfilled layers

XPS was used to monitor the chemical changes occurring on Au substrates functionalized with PEG-COOH upon exposure to OEG₃ or R molecules. The respective relative elemental and chemical species calculated from survey and high resolution XPS scans are summarized in Table 1. The substrate photoelectrons are attenuated by the overlayer and hence the substrate signal has an inverse relationship with overlayer coverage. The Au 4f signal was used to obtain quantitative data on the addition of molecules to the PEG-COOHₙₐₜ. The Au 4f signal of PEG-COOHₙₐₜ+OEG₃ is 3.6% lower than that of PEG-COOHₙₐₜ, indicating that OEG₃ molecules have added to the PEG-COOHₙₐₜ. From the chemical structure of OEG₃ and R molecules it can be observed that they have lower O/C and higher alkane (285.0 eV)/PEG (286.7 eV) [46] ratios when compared to PEG-COOH and hence any addition during backfilling results in a corresponding change in the detected O/C and alkane/PEG ratios.
CHAPTER 3 – Results and discussion

Figure 2. XPS high resolution spectra for the SH-PEG-COOH<sub>SAM</sub>, SH-PEG-COOH<sub>SAM+SH-R</sub> and SH-PEG-COOH<sub>SAM+SH-R-OEG<sub>3</sub></sub> surfaces. (a) shows C 1s spectra, (b) shows S 2p spectra.
Table 1. Summary of XPS results. Au % - relative atomic percentage of Au. O/C - ratio of relative atomic percentage oxygen and carbon. Alkane (285.0 eV)/PEG (286.7 eV) - ratio of alkane and PEG components in C 1s high resolution spectra. % S - relative atomic percentage of S. % bound S - percentage of bound sulfur from components in S 2p high resolution spectra.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PEG-COOH&lt;sub&gt;SAM&lt;/sub&gt;</th>
<th>OEG3-SAM</th>
<th>R&lt;sub&gt;SAM&lt;/sub&gt;</th>
<th>PEG-COOH&lt;sub&gt;SAM +R&lt;/sub&gt;</th>
<th>PEG-COOH&lt;sub&gt;SAM + OEG3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au %</td>
<td>39.0±0.4</td>
<td>32.6±0.4</td>
<td>55.3±2.4</td>
<td>41.6±0.5</td>
<td>36.2±0.5</td>
</tr>
<tr>
<td>O/C</td>
<td>0.49±0.03</td>
<td>0.25±0.01</td>
<td>0.0±0.0</td>
<td>0.40±0.01</td>
<td>0.42±0.01</td>
</tr>
<tr>
<td>Alkane/PEG</td>
<td>0.1±0.0</td>
<td>0.8±0.0</td>
<td>21.0±0.0</td>
<td>0.4±0.0</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>S %</td>
<td>2.1±0.2</td>
<td>1.5±0.2</td>
<td>3.2±0.4</td>
<td>2.1±0.2</td>
<td>1.9±0.6</td>
</tr>
<tr>
<td>% bound S</td>
<td>78.3±0.7</td>
<td>80.7±1.0</td>
<td>88.1±1.0</td>
<td>81.8±1.0</td>
<td>79.9±0.7</td>
</tr>
</tbody>
</table>

The alkane/PEG ratio was calculated from the peak areas of components corresponding to binding energies of 285.0 eV (alkane) and 286.7 eV (PEG) obtained from the high resolution C 1s spectra shown in Figure 2a. Addition of OEG3 is further supported by a decrease in O/C ratio from 0.49 to 0.42 and an increase in alkane/PEG ratio from 0.1 to 0.3 as shown in Table 1. The PEG-COOH<sub>SAM +R</sub> system exhibits a decrease in O/C ratio from 0.49 to 0.40 and an increase in alkane/PEG ratio from 0.1 to 0.4 similar to PEG-COOH<sub>SAM +OEG3</sub> except for the Au signal which increased by 2.6 %. The increase in substrate signal indicates a decrease in overlayer thickness that could be caused by desorption of some PEG-COOH molecules during backfilling by R molecules. Based on the O/C ratios obtained from XPS for PEG-COOH<sub>SAM</sub>, OEG3-SAM, and R<sub>SAM</sub>, a change in O/C ratio as a function of relative coverage of PEG-COOH and OEG3 or R was plotted assuming a linear relationship (Figure 3). By correlating the experimentally obtained values of the O/C ratios for PEG-COOH<sub>SAM +OEG3</sub> and PEG-COOH<sub>SAM +R</sub>, we estimate the coverage of OEG3 or R on the backfilled PEG-COOH<sub>SAM</sub> surface to be 29% and 18%, respectively. It must be noted that this estimation assumes that the intermolecular spaces in PEG-COOH<sub>SAM</sub> ideally are free from any contamination, which in real laboratory conditions is not the case and thus we overestimate the number of added molecules. Here, however, we mainly focus on the relative differences rather than the absolute values.
Figure 3. Relative coverage of PEG-COOH and R or OEG₃ on backfilled surfaces determined by correlating the XPS O/C ratios (X axis) to a plot obtained by extrapolating values of O/C ratio for 0% and 100% relative coverage (Y axis). The data points ▲ and + represent the experimentally obtained O/C ratios for PEG-COOHₙₐₘ₊OEG₃ and PEG-COOHₙₐₘ₊R, respectively.

Despite the greater number of OEG₃ molecules being added to the PEG-COOHₙₐₘ, the decrease in carboxyl peak area (Table 1) in the C 1s spectra of PEG-COOHₙₐₘ₊OEG₃ and PEG-COOHₙₐₘ₊R are comparable. This suggests that desorption of PEG-COOH is lower when OEG₃ is added in comparison to when R is added. It is known that the relative atomic percentage of S from SAMs of PEG thiol depends not only on the stoichiometric ratio but also on the extent of dehydration and collapse of brushes during grafting of chains as described by Unsworth et al. [150]. The amount of S in PEG-COOHₙₐₘ was 2.1 %, which is higher than the corresponding stoichiometric ratio of <1%, very similar to what was reported by Unsworth et al. [150] with hydroxyl terminated 2 kDa PEG thiol molecules grafted under ‘cloud point’ conditions. Though R and OEG₃ molecules have S/C ratios of 0.11 and 0.045, respectively, which is higher than the ratio for PEG-COOHₙₐₘ (0.008), the backfilling of PEG-COOHₙₐₘ with either R or OEG₃ did not significantly change the relative atomic percentage of S (Table 1). Hence the S content was not helpful in providing additional information about the backfilling process. The percentages of bound thiol on functionalized surfaces were determined as described above in the methods section chapter 2. The amount of bound
sulfur was found to increase by a small extent (increase of 3.5%) upon backfilling PEG-COOH$_{\text{SAM}}$ with R, and for OEG$_3$ it increased by 1.6%. This small increase in bound sulfur could be due to Au-thiol bond formation of backfilled molecules, also indicating that a significant proportion of added molecules have chemisorbed to the gold surface and not physically adsorbed. The curve-fitted high resolution S 2p spectra of various samples are shown in Figure 2b.

**B. Arrangement of backfilled molecules**

As discussed earlier, the backfilling process could result in a mixed monolayer with two possible arrangements namely homogeneously mixed layers or islands. Contact angle based surface energy measurements were used to study the arrangement of backfilled molecules. Contact angle measurements have a very high surface sensitivity (<1nm) [137] and allow the determination of the surface energy of material surfaces [138]. The components of surface tension can be calculated using the contact angle values of three different liquids. The values of contact angles for various surfaces are shown in Table 2, and their respective surface tension components; Lifshitz van der Waals ($\gamma_S^d$), electron acceptor ($\gamma_S^+$), electron donor ($\gamma_S^-$) components are shown in Table 3.

**Table 2.** Contact angles for water, formamide and $\alpha$-bromonaphthalene measured on the SH-PEG-COOH$_{\text{SAM}}$, SH-PEG-COOH$_{\text{SAM}}$+SH-R, SH-PEG-COOH$_{\text{SAM}}$+ SH-R-OEG$_3$, SH-R-OEG$_3$-SAM, SH-R surfaces.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water</th>
<th>Formamide</th>
<th>Bromonapthalene</th>
</tr>
</thead>
<tbody>
<tr>
<td>OEG$_3$-SAM</td>
<td>24.3° ± 1.6</td>
<td>13.6° ± 0.5</td>
<td>11.9° ± 0.4</td>
</tr>
<tr>
<td>R$_{\text{SAM}}$</td>
<td>100.3° ± 1</td>
<td>85.3° ± 2.6</td>
<td>48.5° ± 2.6</td>
</tr>
<tr>
<td>PEG-COOH$_{\text{SAM}}$</td>
<td>25° ± 1.7</td>
<td>9.6° ± 1.1</td>
<td>4.8° ± 0.7</td>
</tr>
<tr>
<td>PEG-COOH$_{\text{SAM}}$+R</td>
<td>34.3° ± 0.7</td>
<td>16.8° ± 1</td>
<td>7.2° ± 0.9</td>
</tr>
<tr>
<td>PEG-COOH$_{\text{SAM}}$+OEG$_3$</td>
<td>26.9° ± 1.6</td>
<td>12.3° ± 1.1</td>
<td>7° ± 0.9</td>
</tr>
</tbody>
</table>

The hydrophobicity of a surface is determined by its $\gamma_S^d$ value, with more hydrophobic surfaces having lower values. Since alkane thiol molecules are hydrophobic a SAM of alkane thiol would have a lower $\gamma_S^d$ value than PEG or OEG based SAMs and this is apparent for the
values determined for R_SAM and OEG3-SAM (Table 3). If alkane thiol molecules form islands instead of being homogeneously mixed during the backfilling of the PEG-COOH_SAM, then the $\gamma_d$ value of PEG-COOH_SAM+R would significantly decrease. The $\gamma_d$ value for PEG-COOH_SAM+R is 44.0 mJ/m$^2$ and is virtually the same as that of PEG-COOH_SAM indicating that hydrophobic islands are not present. The $\gamma_s^-$ value of a surface gives a quantitative idea about the presence of electron donor groups, with the greater the value of $\gamma_s^-$ the higher the number of surface electron donor groups. PEG and OEG have oxygen atoms and thus have high $\gamma_s^-$ values when compared to the alkane thiol (Table 2). Backfilling PEG-COOH_SAM with R results in a 7 mJ/m$^2$ decrease in $\gamma_s^-$ indicating that a significant number of PEG-COOH molecules have desorbed from the surface. The values for $\gamma_d$ and $\gamma_s^-$ for PEG-COOH_SAM after addition of OEG3 do not change significantly since both molecules have very similar values and could not easily be followed by contact angle measurements.

Table 3. Surface tension components: the Lifshitz van der Waals component ($\gamma_d$), electron acceptor component ($\gamma_s^+$), and electron donor component ($\gamma_s^-$) determined from the surfaces of OEG3-SAM, R_SAM, PEG-COOH_SAM, PEG-COOH_SAM+R and PEG-COOH_SAM+OEG3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\gamma_d$ mJ/m$^2$</th>
<th>$\gamma_s^+$ mJ/m$^2$</th>
<th>$\gamma_s^-$ mJ/m$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OEG3-SAM</td>
<td>43.45±0.06</td>
<td>0.85±0.05</td>
<td>45.66±1.49</td>
</tr>
<tr>
<td>R_SAM</td>
<td>30.67±1.27</td>
<td>0.01±0.01</td>
<td>0.69±0.26</td>
</tr>
<tr>
<td>PEG-COOH_SAM</td>
<td>44.24±0.04</td>
<td>0.92±0.05</td>
<td>43.97±1.56</td>
</tr>
<tr>
<td>PEG-COOH_SAM+R</td>
<td>44.05±0.08</td>
<td>0.96±0.03</td>
<td>36.58±0.80</td>
</tr>
<tr>
<td>PEG-COOH_SAM+OEG3</td>
<td>44.07±0.1</td>
<td>0.90±0.05</td>
<td>42.90±1.59</td>
</tr>
</tbody>
</table>

C. Arrangement of molecules in mixed layer studied by AFM

QNM-AFM has a very high lateral resolution and has been used to characterize the surface morphology of the different SAM layers with the aim to check the presence of phase separated domains. With the use of the peak forces less than 1 nN, QNM AFM is able to map mechanical properties of surfaces quantitatively while simultaneously capturing topographic images with the same resolutions as traditional tapping mode AFM [146][147]. All QNM-AFM measurements were performed in MQ. The topography and modulus images of Au surfaces
and various SAMs are shown in Figure 4, and the corresponding surface roughnesses are summarized in Table 4.

**Table 4.** Normalized surface roughness (to Au) values for OEG\textsubscript{3-SAM}, R\textsubscript{SAM}, PEG-COOH\textsubscript{SAM}, PEG-COOH\textsubscript{SAM}+R and PEG-COOH\textsubscript{SAM}+ OEG\textsubscript{3}. $S_{\text{SAM}}/S_{\text{Au}}$ stands for ratio of the means of the two surface roughness; $S_{\text{Au}} = 0.48\pm0.02$nm

<table>
<thead>
<tr>
<th>Sample</th>
<th>Roughness (Sq) (nm)</th>
<th>$S_{\text{SAM}}/S_{\text{Au}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-COOH\textsubscript{SAM}</td>
<td>0.69±0.04</td>
<td>1.44</td>
</tr>
<tr>
<td>OEG\textsubscript{3-SAM}</td>
<td>0.63±0.04</td>
<td>1.31</td>
</tr>
<tr>
<td>R\textsubscript{SAM}</td>
<td>0.21±0.02</td>
<td>0.44</td>
</tr>
<tr>
<td>PEG-COOH\textsubscript{SAM}+OEG\textsubscript{3}</td>
<td>0.81±0.05</td>
<td>1.69</td>
</tr>
<tr>
<td>PEG-COOH\textsubscript{SAM}+R</td>
<td>0.66±0.06</td>
<td>1.38</td>
</tr>
</tbody>
</table>

**Figure 4.** Topography and Young’s modulus images recorded by QNM-AFM measurements in MQ water. (a, a’) Au substrate, (b, b’) PEG-COOH\textsubscript{SAM}, (c, c’) OEG\textsubscript{3-SAM}, (d,
d’) $R_{SAM}$, (e, e’) $PEG-COOH_{SAM+OEG_3}$, and (f, f’) $PEG-COOH_{SAM+R}$. The scan area of each image is 1µm×1µm.

The topography of the bare Au surface (Figure 4a) shows Au grains without any additional features on top of the grains. The recorded nanomechanical map of a Au surface (Figure 4a’) demonstrates the homogeneous Young’s modulus distribution as the whole image has nearly the same range of stiffness showing the same color contrast. Comparing the topography images of modified surfaces with the bare Au, one can clearly see the additional particle-like features on all the modified surfaces (Figure 4b-f). In addition, the modulus images of the modified surfaces show that many of the grain boundaries are slightly invisible due to the SAMs. Furthermore, the surface roughness has been changed dramatically after modification. Hence these results prove the successful formation of the SAMs. Interestingly, the modulus of the modified surfaces also represents the surface modulus variations, because of the slightly different color contrast (Figure 4b’-4f’). The small variations in material properties observed in elastic modulus measurements are particularly useful in the analysis of heterogeneous surfaces. However most importantly, phase separations were not observed in any of images of the backfilled surfaces, indicating that they are homogeneous. Hence, it is concluded that the SH-R-OEG$_3$ or SH-R molecules form homogeneous mixed monolayers upon addition to SH-PEG-COOH$_{SAM}$ rather than separate domains. The surface roughness of $PEG-COOH_{SAM}$ increases by 0.12 nm upon exposure to OEG$_3$ molecules. Lee et al. [154] showed that the proportion of DNA molecules in upright orientation in a SAM of thiolated single strand DNA on gold increased upon exposure to OEG terminated alkane thiol molecules. An increase in proportion of PEG molecules in upright orientation could explain the increase in surface roughness of $PEG-COOH_{SAM}$ upon exposure to OEG$_3$. Lee et al. [162] showed that addition of alkylthiol molecules onto SAM of thiolated single strand DNA on gold increased the fraction of DNA molecules in upright orientation. Once should expect a similar effect during the addition of R onto $PEG-COOH_{SAM}$, but the surface roughness of $PEG-COOH_{SAM+R}$ in fact decreases by 0.03 nm (decrease not significant due to high deviation). It must be noted that the addition of R caused more desorption of $PEG-COOH$ molecules during backfilling when compared to that observed during the addition of OEG$_3$. Thus desorption of $PEG-COOH$ molecules during the addition of
R could be the reason behind the surface roughness of PEG-COOH\textsubscript{SAM} +R not being higher than PEG-COOH\textsubscript{SAM}.

D. Resistance of backfilled PEG-COOH surface towards protein adsorption and bacterial attachment

Resistance towards non-specific adsorption can be tested using simple model systems such as single protein solutions. Examples have included collagen [163] and bovine serum albumin [132]. More rigorous tests are conducted with serum [132] or microbial cell suspensions [42]. Once the surfaces have been assessed by model testing techniques, their performance in real environments such as a food processing plant, as coatings on clinical implants or marine waters could be studied. The non-fouling properties of the PEG-COOH\textsubscript{SAM} and mixed surfaces were evaluated by serum adsorption and bacterial attachment studies. Addition of smaller OEG chains in theory increases the graft density of protein resistance molecules within the layer of diffuse PEG-COOH\textsubscript{SAM} molecules and hence is expected to improve its resistance towards non-specific adsorption. QCM and XPS have been employed to quantitatively compare the serum adsorption resistance properties of the SAMs. The frequency shift after serum exposure in QCM is directly proportional to the amount of protein adsorbed [132], and the relative atomic percentage of nitrogen from XPS also enables a quantitative estimation of the amount of adsorbed protein [163]. Representative QCM kinetic plots are shown in Figure 5. In Figure 6 we show the data points corresponding to various samples studied, where the X-axis shows the percentage of nitrogen (% N) obtained from XPS and the Y-axis shows the frequency shift from the QCM data. Using the N atomic percentage and the frequency shift in QCM, the amount of protein adsorbed was determined as shown in Table 5. Upon serum exposure the resonance frequency decreases by 7 Hz for the PEG-COOH\textsubscript{SAM}+OEG\textsubscript{3} surface, compared to both PEG-COOH\textsubscript{SAM} and PEG-COOH\textsubscript{SAM}+R surfaces, which exhibit a frequency shift of 15 Hz. The lower frequency shift for PEG-COOH\textsubscript{SAM}+OEG\textsubscript{3} proves that OEG\textsubscript{3} addition improves the non-fouling abilities of PEG-COOH\textsubscript{SAM} surfaces. It is also speculated that protein adsorption would be lower if the PEG thiol molecules used did not have a carboxyl end-group, since carboxyl groups under physiological pH would interact electrostatically with serum proteins, thus facilitating higher
adsorption. Figure 6 shows nitrogen contents on the X-axis, where the PEG-COOH$_{\text{SAM}}$+OEG$_3$ surface has 2% N compared to PEG-COOH$_{\text{SAM}}$ and PEG-COOH$_{\text{SAM}}$+R surfaces both showing approximately 5% N, which follows the same trend as seen with shift in resonance frequencies obtained using QCM. The OEG$_3$-SAM had the highest ability to resist serum adsorption which agrees with previous studies with OEG based SAMs [46]. The hydration factor was calculated by taking the ratio of wet mass and dry mass from QCM and XPS respectively [164]. The adsorption of proteins onto hydrophobic surfaces would increase the chances of denaturation of the molecule thereby making it more rigid and less hydrated [46]. The hydration factor values calculated are in the following order PEG-COOH$_{\text{SAM}}$+OEG$_3$ > PEG-COOH$_{\text{SAM}}$ $\cong$ PEG-COOH$_{\text{SAM}}$+R. The higher hydration factor of OEG backfilled SAM could be due to the higher hydration and lower rigidity of adsorbed serum protein molecules during the exposure to serum or a different type(s) of proteins adsorbing to each surface from the complex serum. This further supports the speculation the PEG SAM backfilled OEG would be ideal surface for immobilization of bioactive molecules. Gold was used as reference sample which had a hydration factor of 1.8, least among all samples, indicating that the major proportion of proteins adsorbed onto gold are rigid due to higher surface hydrophobicity.

Table 5. Hydration factor for PEG-COOH$_{\text{SAM}}$, PEG-COOH$_{\text{SAM}}$+R and PEG-COOH$_{\text{SAM}}$+OEG$_3$ calculated from the ratio of QCM based wet thickness and XPS based dry thickness as described by Ayyoob et al.[164].

<table>
<thead>
<tr>
<th></th>
<th>QCM ng/cm$^2$</th>
<th>XPS ng/cm$^2$</th>
<th>Hydration factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG$_{\text{SAM}}$</td>
<td>36.7</td>
<td>12.7</td>
<td>2.9</td>
</tr>
<tr>
<td>PEG$_{\text{SAM}}$+OEG</td>
<td>19.5</td>
<td>5.0</td>
<td>3.9</td>
</tr>
<tr>
<td>PEG$_{\text{SAM}}$+R</td>
<td>36.4</td>
<td>13.5</td>
<td>2.7</td>
</tr>
<tr>
<td>Gold</td>
<td>126</td>
<td>67.7</td>
<td>1.8</td>
</tr>
</tbody>
</table>
CHAPTER 3 – Results and discussion

**Figure 5.** Representative QCM plots showing the change in frequency upon exposure to 10 % FBS, 37°C 750 µl, 100 µl/min on: Au, PEG-COOH_{SAM}, PEG-COOH_{SAM}+R, PEG-COOH_{SAM}+OEG₃, R₃SAM and OEG₃-SAM. The time points of serum exposure and PBS rinse are indicated with arrows.

**Figure 6.** Quantification of N% (by XPS) and resonance frequency change (by QCM) after exposure of PEG-COOH_{SAM}, PEG-COOH_{SAM}+R and PEG-COOH_{SAM}+OEG₃, R₃SAM and OEG₃-SAM surfaces to 10% FBS at 37°C.
The surfaces were tested for bacterial attachment using a *Staphylococcus aureus* strain. The results of bacterial attachment studies are shown in Figure 7. Clearly, the PEG-COOH_{SAM}+OEG_{3} (4.2 × 10^7 cells/cm²) surface is better at resisting bacterial attachment when compared to PEG-COOH_{SAM} (7.2 × 10^7 cells/cm²) and PEG-COOH_{SAM}+R (9.2 × 10^7 cells/cm²). Hence it can be concluded that adding OEG_{3} molecules improve the non-fouling properties of the PEG-COOH_{SAM}. Though the OEG_{3-SAM} has the least bacterial attachment and serum adsorption, we propose that PEG-COOH_{SAM}+OEG_{3} system would be a better system for applications involving immobilization of bioactive molecules such as enzymes and further advantages of PEG-COOH_{SAM}+OEG_{3} will be discussed in the next section. The bacterial attachment studies performed here are short term studies and a comprehensive study would involve the study of the time biofilm formation starting from the initial adhesion, followed by microcolonies formation and then the maturation phases I, II [151]. Thus the preliminary bacterial initial attachment studies give an idea about the effect of backfilling on the resistance of surfaces towards initial attachment of bacterial cells.

![Bacterial adhesion study](image)

*Figure 7. Attachment studies using Staphylococcus aureus to PEG-COOH_{SAM}, PEG-COOH_{SAM}+OEG_{3}, OEG_{3-SAM}, R_{SAM}.*
E. Discussion

We have studied mixed layers of OEG$_3$ and PEG-COOH$_{SAM}$ using XPS, contact angles and AFM. The contact angle results support the notion that phase separated surface arrangement of the two molecules does not occur proving that homogeneous mixed layers are formed. There have been some reports on grafting 2 kDa PEG thiol chains. For example Unsworth et al. [150] reported a maximum graft density of 0.58 chains/nm$^2$ using methoxy capped 2 kDa PEG thiol chains under ‘cloud point’ conditions, and Tokumitsu et al. [165] reported a maximum graft density of 3.6 chains/nm$^2$ using 2 kDa PEG conjugated to undecane thiols. From our XPS data we have calculated a graft density of PEG-COOH$_{SAM}$ to be 1.0 chain/nm$^2$. Thus, the PEG-COOH chains in PEG-COOH$_{SAM}$ have a density that is not close-packed and there exists sufficient space for the smaller OEG molecules to fill the gaps, although in a good solvent the PEG is likely to extend and form highly mobile chains [150].

The graft density of close packed SAM of PEG brush is approximately 4.5 chains//nm$^2$ assuming a cross-sectional area of PEG molecule in helical conformation to be 22 Å$^2$ as reported by Harder et al. [46]. There are several reports on protein resistant PEG SAMs in the literature. Dalsin et al. measured the protein resistance of DOPA functionalized 5 kDa PEG SAMs on titanium dioxide surface [166]. They reported serum asorption <1ng/cm$^2$ for PEG surface with an approximate graft density of 3.2 chains/nm$^2$, and that graft density corresponded to brush conformation. A similar study was reported by Pasche et al. who used PLL-g-PEG SAMs to create surfaces with PEG brushes that showed serum adsorption as low as <1ng/cm$^2$ on nobium oxide surface [167].

The size of protein molecules is one of the most important parameters determining the qualitative and quantitative aspects of adsorption on polymer coated surfaces. If the SAM of PEG on a surface is exposed to protein molecules that are smaller than the interchain distances, then the PEG SAMs fail to effectively resist protein adsorption. Uchida et al. [115] performed protein adsorption studies using a range of proteins with varying molecular weight on mixed monolayers of 5 and 2 kDa PEG thiol on gold using backfilling method. They demonstrated these backfilled monolayer could resist the adsorption of protein in the $M_W$ range 400Da – 340 kDa (surface protein <1ng/cm$^2$). The carboxyl terminated PEG chains chosen in experimental studies performed in this chapter
would possess a negative charge at physiological pH. Carboxyl capped PEG molecules were used since it is intended to use them for immobilization of antibacterial enzymes in future studies. These molecules are expected to contribute to the surface negative charge. It is suspected that the non-fouling properties would be better with hydroxyl or methoxy capped PEG molecules, which are neutrally charged and thus minimize electrostatic interactions. The mixed PEG-COOH$_{\text{SAM}}$+OEG$_3$ system is anticipated to be an ideal platform for immobilization of bioactive molecules, since the OEG maximizes the non-fouling potential while the longer PEG chains would confer higher mobility and thus improved bioactivity capabilities to the immobilized molecule [161]. There have been reports on bacterial attachment studies on OEG [168] and PEG [42] surfaces. The attachment studies with *Staphylococcus aureus* involve different incubation times compared to that used by Nejadnik et al. [42], and Ostuni et al. [168], who used a counting technique involving removal of attached cells by sonication followed by counting the colony forming units on culture plates that introduces possibility of 10 fold error in estimation as mentioned by authors. Hence the absolute values the bacterial attachment studies found here could not be compared with these earlier reported studies. Based on the studies of backfilled PEG-COOH$_{\text{SAM}}$ surfaces, it was found that OEG$_3$-SAM are better at resisting fouling when compared to all other surfaces studied in the project. Otsuka et al. demonstrated that the biorecognition efficiency benefit from longer PEG chains is due to higher mobility [161]. Thus the combination of PEG-COOH$_{\text{SAM}}$ backfilled with OEG$_3$ will be an ideal support for immobilizing bioactive molecules because the longer PEG molecule would confer higher mobility and backfilled OEG molecules would improve ability to resist non specific adsorption. In Chapter 4 preliminary results are presented of relating to immobilizing antifouling enzyme molecules (subtilisin) on backfilled PEG-COOH$_{\text{SAM}}$+OEG$_3$ surfaces with further understandings of the effect of backfilled OEG molecules on the activity and performance of enzyme molecules.

**IV. SUMMARY AND CONCLUSION**

Backfilling of a PEG-COOH$_{\text{SAM}}$ with alkane and oligo ethylene glycol terminated thiol molecules was investigated. From XPS and contact angle studies it was shown that R molecules form mixed layers and the addition process is accompanied by some desorption of
the PEG-COOH molecules. OEG\(_3\) molecules, upon addition, also formed mixed layers but less desorption of the PEG occurs, when compared to addition of R molecules. The non-fouling properties of various surfaces were compared, and it was shown that the PEG-COOH\(_{\text{SAM}}\)+OEG\(_3\) surface was found to be better than the PEG-COOH\(_{\text{SAM}}\) surface with respect to resisting serum adsorption and bacterial attachment. Thus, the strategy involving backfilling using OEG terminating alkane thiols is indeed capable of improving the non-fouling properties of layer of PEG chains of significant initial graft density.
CHAPTER 4

Immobilization of active subtilisin to mixed poly(ethylene glycol) and oligo(ethylene glycol) layers

A. Introduction

Antifouling enzymes are naturally occurring biodegradable biocatalysts that have the potential to be an efficient and environmentally friendly strategy for preventing biofouling [4, 34, 169]. The usefulness of an enzyme in a specific application depends on the reaction it catalyzes. The choice of appropriate enzyme for prevention of microbial biofilm formation processes requires that the enzyme using its catalytic reaction capability interferes with biofilm formation process. The steps involved in microbial biofilm formation processes were discussed in Chapter 1. The extracellular polymeric substance (EPS) has been shown to be a very important in microbial adhesion, colonization and biofilm formation [20, 51-53]. The EPS mainly consists of polysaccharides [51], extracellular DNA [20] and glycoproteins. Disturbing the integrity of EPS by damaging one of the important constituents of EPS renders the microbe incapable of surface adhesion and/or biofilm formation [20, 51]. According to Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), enzymes can be classified based on the types of reactions they catalyze [171]. Various classes of enzymes are given in Table 1. Among the classes of enzymes mentioned in Table 1, hydrolases and lyases are most relevant when it comes to non-toxic antifouling applications. Since the enzymes do not kill organisms and get degraded easily technologies based on enzymes are environmentally benign [170]. Many examples of enzyme based prevention or treatment of biofilms can be found in literature. Lysozyme, trypsin, pepsin, subtilisin, α-amylase, DNase I and alginate lyase have been demonstrated to interfere with either the biofilm formation process or damage an existing biofilm [20, 34, 51, 57, 169, 172, 173]. The modes of action of different groups of enzymes are listed in Table 2. Apart from the EPS damaging enzymes listed, there are a few other protein molecules which are capable of interfering with biofilm formation. Intercellular communication in biofilm occurs through release of quorum sensing molecules such as the N-acyl homoserine lactones (AHL) [174]. Enzymes
such as AHL acylase interrupt the quorum sensing mechanism thereby stopping further development of biofilm [175]. Lactoferrin is a mammalian protein relevant to immune system which limits bacterial infections by sequestering iron from the immediate surrounding environment, and use of this protein has been demonstrated to slow down the biofilm formation process [56]. The work presented in this chapter focuses on investigating the antifouling properties of backfilled PEG-COOH SAMs immobilized with subtilisin, which is a widely used serine protease.

**Table 1.** Various classes of enzymes and their corresponding function. The classification is in accordance with NC-IUBMB.

<table>
<thead>
<tr>
<th>Enzyme class</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 1 Oxidoreductases</td>
<td>catalyze red-ox reactions</td>
</tr>
<tr>
<td>EC 2 Transferases</td>
<td>catalyze the transfer of a chemical group from donor to acceptor</td>
</tr>
<tr>
<td>EC 3 Hydrolases</td>
<td>catalyze the hydrolysis of various bonds</td>
</tr>
<tr>
<td>EC 4 Lyases</td>
<td>catalyze cleaving bonds by means other than by hydrolysis or oxidation</td>
</tr>
<tr>
<td>EC 5 Isomerases</td>
<td>catalyze isomerization</td>
</tr>
<tr>
<td>EC 6 Ligases</td>
<td>catalyze ligation of molecules</td>
</tr>
</tbody>
</table>

**Table 2.** Mode of action for different groups of enzymes used in biofilm prevention or treatment [20, 34, 51, 57, 169, 172, 173].

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin, pepsin, subtilisin</td>
<td>Peptide bond cleavage</td>
</tr>
<tr>
<td>Lysozyme, α-amylase, alginate lyase</td>
<td>Glycosidic cleavage</td>
</tr>
<tr>
<td>Dnase I</td>
<td>Phosphodiester cleavage</td>
</tr>
<tr>
<td></td>
<td>Extracellular DNA damage</td>
</tr>
</tbody>
</table>
Proteases constitute the largest volume of enzymes used in industrial applications. Subtilisin enzyme accounts for 35 percent of the total enzyme market, with an approximate annual production of 500 tones [176, 177]. Subtilisin is one of the most well studied serine proteases used in a number of applications such as food processing, leather treatment, detergents, waste management, pharmaceuticals, silver recovery and organic synthesis [178-180]. Subtilisin has been shown to have antifouling properties against several species of bacteria [169] and it works by cleaving bacterial cell membrane proteins consequently inhibiting adhesion to surfaces. Microbes use special adhesion organelles such as pili [181] and proteolytic or glycosidase enzymes capable of damaging these adhesion organelles and thus prevent the microbes from adhering to surfaces. Due to the wide spread usage in industrial applications and antifouling capabilities of serine proteases, subtilisin was chosen as a model enzyme to perform immobilization studies on mixed SAM of PEG and OEG created by backfilling as described in Chapter 3. Preliminary experiments with BSA and lysozyme were also performed to compare the results of immobilization studies with those obtained using subtilisin.

Enzymes can be used either as suspensions or in immobilized form [182]. Using immobilized form of enzyme has several advantages over a suspended enzyme such as easy recovery and reusability, but on the down side the immobilization step can lead to a loss of enzymatic activity due to conformational changes and/or denaturation [182, 183]. Protein molecules adsorb onto surfaces through van der Waals, electrostatic, covalent and dispersion interactions, with dispersive or hydrophobic-hydrophobic interactions being thermodynamically most significant when it comes to protein denaturation [184-186]. Strategies used to improve or retain the activity of immobilized subtilisin can be broadly classified into two categories. The most popular one being enzyme structure modification using protein engineering techniques such as addition of affinity tags, sulfhydryl and site directed mutagenesis [183, 187, 188]. The second category includes strategies which involve substrate (surface) modification using polymers that prevent protein denaturation [188-190]. The changes introduced by protein engineering techniques improve the overall performance of an immobilized enzyme by making it thermally more stable towards denaturation or providing specific conformational orientation to the enzyme molecule. The strategy involving
hydrophilic polymer coatings on substrates minimizes the loss of enzyme activity upon immobilization by presenting a steric barrier between the enzyme molecule and the substrate surface thus preventing the unfavorable interactions such as hydrophobic-hydrophobic interactions that denature the enzyme or protein molecules [188-190]. PEG is one of the most widely used hydrophilic nonfouling polymers for reasons discussed in Chapter 1. There are several strategies to maximize the graft density of PEG coatings via ‘grafting to’ techniques such as ‘cloud point grafting’ [41] and backfilling [115]. In Chapter 3 we presented results of detailed surface characterization of SAMs formed by backfilling PEG thiol layers with OEG thiol molecules. These backfilled surfaces are an ideal support for immobilizing bioactive molecules such as enzymes as they would benefit from longer PEG chains allowing higher mobility and potentially enhanced biorecognition [161] while avoiding non-specific adsorption of proteins [153]. In this chapter, the focus was to immobilize subtilisin on the surfaces of carboxyl terminated PEG-COOH and OEG-COOH thiols assembled on gold with comparisons being made to PEG-COOH backfilled with OEG thiols on gold. The hypothesis is that immobilization of enzyme on PEG-COOH SAM backfilled with OEG thiols would result in minimum non-specific adsorption and higher enzyme activity when compared to PEG-COOH SAMs.

The covalent immobilization of biomolecules onto surfaces requires that they have appropriate functional groups present in order for the coupling reactions to take place. Non-covalent immobilization is also used but covalent coupling often provides greater stability [191]. Some of the widely used surfaces such as titanium, steel, and polymeric materials do not have the appropriate reactive groups required to react with biomolecules, and this limitation can be overcome by functionalizing the surfaces, for example, by oxidation to introduce hydroxyl or carboxyl groups; coating with SAMs with appropriate tail groups [84, 86]. There are several well known reactions used for coupling purposes such as carboxyl-amine, aldehyde-amine, thiol-thiol, amine-thiol, hydroxyl-amine, amine-amine, thiol-carbonyl, and thiol-maleimide. A host of other reactions are possible depending on the availability of functional groups, reaction efficiencies and conditions required for reactions [84, 87]. The amino acids relevant to immobilization of proteins are listed in Table 3. Aspartic acid and glutamic acid residues have a carboxylic acid group that can be used to couple the protein by
forming linkages such as ester (with hydroxyl), thioester (with thiol), amide (amine). Often these reactions require activation of the carboxyl to form a active ester intermediate, which acts as a better electrophile when compared to the carboxyl anion [87]. Lysine residues have a primary amine that can react either directly with alkyl halides to give alkyl substituted amines or activated carboxyl or acyl group resulting in an amide bond formation [87]. In this project we choose the amine based amide formation to couple the proteins onto PEG SAM whose tail groups were an active ester intermediate. The yield of a reaction between a nucleophile (amine) and an electrophile (carboxylic ester intermediate) depends on reaction conditions such as medium temperature, state of protonation of amine (depending on pl, pH) [87].

**Table 3.** Properties of subtilisin, lysozyme and BSA relevant to the immobilization experiments. The $M_W$, the number of important residues, theoretical relative atomic percentages of C, N, O, and S were found using ExPASy ProtParam tool [192]. The amino acid sequences were obtained from NCBI protein database [193]. The pl values of proteins were provided by supplier Sigma Aldrich, Denmark.

<table>
<thead>
<tr>
<th></th>
<th>Subtilisin</th>
<th>Lysozyme</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_W$ (Kda)</td>
<td>27.2</td>
<td>14.2</td>
<td>69.2</td>
</tr>
<tr>
<td>Carbon (%)</td>
<td>62.1</td>
<td>61.4</td>
<td>63.3</td>
</tr>
<tr>
<td>Nitrogen (%)</td>
<td>17.2</td>
<td>19.2</td>
<td>16.8</td>
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<tr>
<td>Oxygen (%)</td>
<td>20.5</td>
<td>18.4</td>
<td>19.1</td>
</tr>
<tr>
<td>Sulphur (%)</td>
<td>0.3</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>pl (isoelectric pH)</td>
<td>9.4</td>
<td>11.3</td>
<td>5</td>
</tr>
<tr>
<td>Arg (R) no of AA</td>
<td>4</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td>Glu (E) no of AA</td>
<td>5</td>
<td>2</td>
<td>59</td>
</tr>
<tr>
<td>Asp (D) no of AA</td>
<td>9</td>
<td>7</td>
<td>40</td>
</tr>
<tr>
<td>Cys (C) no of AA</td>
<td>0</td>
<td>8</td>
<td>35</td>
</tr>
<tr>
<td>His (H) no of AA</td>
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<td>1</td>
<td>17</td>
</tr>
<tr>
<td>Lys (K) no of AA</td>
<td>9</td>
<td>7</td>
<td>60</td>
</tr>
<tr>
<td>Tyr (Y) no of AA</td>
<td>13</td>
<td>3</td>
<td>21</td>
</tr>
</tbody>
</table>
Water soluble carbodiimide chemistry is often used to catalyze carboxyl reactions with primary amine groups when immobilizing proteins. One example of such amide bond formation is the use of both N-hydroxy succinimide (NHS) and ethylenediamine carbodiimide (EDC), which forms an active ester intermediate as shown in Figure 1 [87, 194, 195]. The next step involves nucleophilic attack on the carbonyl of the active ester adjacent to the amine is also shown in Figure 1. This second step is highly sensitive to the state of ionization of the amine group, since deprotonated amines (neutral) are nucleophilic while protonated amines (positively charged) are not nucleophilic. The state of protonation depends on the pH of reaction medium relative to the isoelectric pH of the protein being immobilized [87]. The pI values of the proteins used in the immobilization experiments in this project are listed in Table 3.

**Figure 1.** The schematic of the mechanism of sulfo-NHS-EDC mediated coupling of enzyme molecules onto carboxyl coating surfaces such as end-capped PEG SAMs [196, 197].

The determination of the extent of activity retained by immobilized enzyme can be
measured using colorimetric assays. In this study the amount of enzyme on the surfaces was quantified using XPS by monitoring changes in the N 1s signal from the nitrogen containing amino acids of enzyme molecules. The activity of enzyme immobilized on the PEG surface was quantified by a colorimetric assay using a chromogenic substrate, which was an enzyme cleavable oligopeptide coupled to para-nitroaniline (pNa) [183]. The intact unhydrolyzed pNa molecule (bound to oligopeptide) has an absorption maximum at 316 nm, whereas the free p-Nitroaniline (pNa) has an absorption maximum at 380 nm. Thus the rate of change of absorbance at 385 nm allows a measure of the hydrolysis of oligopeptides [198]. In general practice absorption is measured at 405 nm instead of 385 nm to minimize the interference due to the absorption peak at 310 nm due to intact unhydrolyzed pNa molecules [198]. The enzyme functionalized surfaces were tested for their ability to resist bacterial adhesion using Staphylococcus xylosus (S. xylosus) and Staphylococcus aureus (S. aureus). The bacteria S. xylosus is a strain of bacteria highly relevant to dairy industry, where there is interest in preventing fouling by our industrial collaborator Arla Foods A/S. S. aureus is a clinically relevant bacterium that is responsible for a number of infections and also responsible for the failure of implants.

II. Materials and Methods

A. Chemicals

Gold surfaces were cleaned the same way as described in Chapter 3. 2 kDa α-carboxyl-ω-thiol poly(ethylene glycol) (PEG-COOH, >98% purity) was purchased from Laysan Bio Inc. (Alabama, USA); hydroxyl-terminated tri (ethylene glycol) undecane thiol (OEG, 99% purity) and carboxy-terminated tetra (ethylene glycol) undecane thiol (OEG-COOH, 99% purity) were purchased from Assemblon (Washington, USA). Subtilisin A (from Bacillus licheniformis), chicken lysozyme (Lyz), bovine serum albumin (BSA), octane thiol (R) (99% purity), SYBR Green II, pNa, phosphate buffered saline (PBS) buffer tablets, 25% ammonium hydroxide (NH₄OH), 30% hydrogen peroxide (H₂O₂) and absolute ethanol were purchased from Sigma Aldrich (Aarhus, Denmark). Chromogenic substrate for subtilisin suc-ala-ala-phe-pNa was purchased from Bachem, Switzerland. Ultrapure MilliQ (MQ) water with the
resistivity of 18.2 MΩ was used for buffers and aqueous ethanolic solutions.

**B. Gold substrate functionalization**

The SAMs of PEG-COOH thiols backfilled with OEG thiols were prepared as described in Chapter 3. Mixed SAMs of OEG and OEG-COOH thiol were prepared by co-adsorption from solutions. The gold slides were incubated in 1mM thiol solutions (appropriate proportions) in ethanolic solutions (99.8%) for 24h. The functionalized slides were rinsed with ethanol to remove any physically adsorbed OEG or OEG-COOH thiol molecules.

![Chemical structures](image)

Figure 2. The chemical structures of thiolated molecules used for functionalizing gold.

The following notations for SAM of PEG and for the backfilled surface:

- OEG + OEG-COOH: mixed OEG and OEG-COOH SAM created by coadsorption
- PEG-COOH+OEG: PEG-COOH SAM backfilled with OEG
- PEG-COOH+R: PEG-COOH SAM backfilled with R

**C. Enzyme immobilization**

The NHS-EDC based coupling of proteins is a well studied reaction. For example
Grainger et al reported the need for a 15 minutes carboxyl activation step using 0.5M NHS-EDC in MQ followed by 30 minutes incubation with an amine containing molecule to perform amide bond based biomolecular immobilization [194, 199]. Optimal NHS-EDC concentrations required for immobilization of subtilisin was determined experimentally by systematically varying the NHS-EDC concentration from 1 mg-10 mg/ml, 10 mg-100 mg/ml, 100 mg-1 g/ml and 500 mg-5 mg/ml in MQ water for 30 minutes. After the NHS-EDC incubation, the activated slides were incubated in 1 mg/ml subtilisin solution in PBS (pH 7.4) for 3h. Based on these findings the OEG/OEG-COOH functionalized surfaces were activated for 30 minutes with a mixed solution of EDC and NHS (50 and 5 mg/ml respectively) at pH 6.0 in MQ water, followed by incubation with 1 mg/ml subtilisin solution for 3h. The enzyme immobilized surfaces were rinsed with 100mM PBS (pH 7.4) to remove loosely bound enzyme molecules prior to characterization and testing. During the NHS-EDC coupling experiments it was observed that air bubbles were formed on PEG-COOH functionalized surfaces. Thus the NHS-EDC concentration had to be lowered. It is known that sulfo-NHS has higher solubility than NHS, thus a lower concentration of sulfo-NHS was used in order to minimize bubble formation. The enzyme immobilization on SAMs of PEG-COOH backfilled with OEG thiols was done the same way as mentioned above, except for lower EDC/Sulfo-NHS (2/0.2 mg/ml) concentration used. Since the carboxyl activation conditions used were different for OEG/OEG-COOH and PEG-COOH systems, we could not make direct comparison between these sets of samples. Control experiments were also performed using denatured subtilisin enzyme. Enzyme denaturation was performed using a PCR machine for heating active enzyme solutions in PBS (pH 7.4) at 90° C for 3h. The heated solution of enzyme was tested for activity, which confirmed that all activity was lost. Since the immobilization condition required for subtilisin immobilization was different from those reported in the literature using other proteins, it was checked if the surfaces used in the project had something to do with the low NHS-EDC concentration required for protein immobilization. Lysozyme and BSA was used to perform immobilization experiments on PEG-COOH SAMs backfilled with OEG thiols using the NHS-EDC concentrations similar to that reported by Grainger et al. [194, 199]. The PEG-COOH slides were activated using EDC-NHS (0.1/0.1 M) for 20 minutes followed by incubation of 1 mg/ml solutions of either lysozyme (PBS pH 7.4) or BSA (acetate buffer pH
4.0) for 30 min. This incubation step was followed by rinsing with PBS and then with MQ. The slides were then dried using a jet of nitrogen gas, followed by XPS analysis.

D. Enzyme quantification by XPS and activity measurement

The quantification of enzymes on the surfaces was done using XPS by monitoring changes in the N 1s signal from the survey spectra. The activity of the immobilized subtilisin on the PEG surfaces was quantified by a colorimetric assay. The colorimetric assay was performed by immersing 1cm×1cm substrates in 12 well microtiter plates containing 2 ml, 5mM suc-alala-ala-phe-pNa solution (HEPES buffer pH 8.0) and measuring the absorbance (at 405 nm) of 2 μl reaction mixture which was pipetted out of the reaction mixture once every 3 minutes. The absorbance was monitored using a nanodrop instrument (NanoDrop 1000, Nanodrop products, Delaware, USA) capable of measuring absorbance of 1-10 μl volumes. The small sampling volume was important in order to ensure that the volume of chromogenic substrate solution did not decrease significantly during the measurement. Based on the measured rate of change of absorbance, the rate of change of pNa concentration was calculated using a calibration curve obtained from absorbance measurements using various concentrations of pNa. The unit of enzyme activity is the enzyme unit (U-μM/min) and is defined as the number of micromoles of substrate modified in one minute [200].

E. Bacterial adhesion assay

Bacterial adhesion assays were performed using two gram positive bacteria, namely, *Staphylococcus xylosus* (*S. xylosus* DSM 20266) and *Staphylococcus aureus* (*S. aureus* ATCC 12598). A starter culture was inoculated from agar plates and grown in 3 ml of 1% tryptic soy broth (TSB) medium in 50 ml conical bottom tube by incubating overnight in a shaker at 37°C. An inoculum prepared by adding 1 ml of starter culture to 100 ml 1% TSB medium was incubated in a shaker at 37°C until cultures were in the late-exponential growth phase (OD600 = 1.0). Cells were then harvested by centrifugation (5 minutes at 3000 x g), washed twice and resuspended in PBS (pH 7.4) and diluted to obtain the required optical density (OD). The slides to be tested for bacterial adhesion were exposed to bacterial suspensions of *S. xylosus*: OD-0.01, incubation time 2h; and *S. aureus*: OD-0.1, incubation
time of 5h, on an orbital shaker at 37°C. The slides were rinsed with PBS and stained with SYBR green dye. Bacterial counting was done by manual counting using a fluorescence microscope. Cells were counted in 109 µm² grids in triplicate with a total of fifteen random positions on each type of surface. Adherent cells were counted manually by fluorescence microscopy using a Zeiss Axiovert 200M epifluorescence microscope (Carl Zeiss GmbH, Jena, Germany) equipped with Zeiss filter set and 10 and 63x oil immersion objective. Student T test was used to assess the statistical significance results during comparison.

III. Results

A. Subtilisin immobilization OEG-COOH/OEG mixed monolayer

i. XPS characterization of the OEG-COOH/OEG mixed surfaces

The optimum NHS-EDC concentration required for immobilization of subtilisin was found experimentally by performing immobilization at varying concentrations of NHS-EDC. Except for the concentration of NHS-EDC, the immobilization of subtilisin on 100% OEG-COOH SAM, and characterization using XPS was done the same way as described in methods section. The results of this study are presented in Table 4. The amount enzyme immobilized was followed using the relative atomic percentage of nitrogen. The maximum enzyme immobilization was achieved using a NHS-EDC concentration of 100 mg-1 g/ml, and achieved a %N of 4.6%. Surprisingly the %N decreased at higher NHS-EDC concentrations (500 mg-5 g/ml). On the other hand the decrease in %N at lower NHS-EDC concentrations is expected since at lower concentrations of NHS-EDC, there would be a decreased number of activated carboxyl groups. Thus the increase in potential sites for immobilization of subtilisin molecules beyond a certain threshold leads to a decrease in the total number of molecules immobilized. To understand the relationship between surface coverage of subtilisin and specific activity, we performed immobilization experiments on surfaces with varying proportions of COOH, created by co-adsorption of mixtures of OEG-COOH and OEG thiols.

Table 4. Results of XPS studies on 100% OEG-COOH SAM immobilized with subtilisin using different NHS-EDC concentrations of 1 mg-10 mg/ml, 10 mg-100 mg/ml, 100 mg-1 g/ml
NHS-EDC Concentration & C rel at% & O rel at% & S rel at% & Au rel at% & N rel at% 
1 mg-10 mg/ml & 48.3±1.2 & 11.7±0.5 & 2.4±0.4 & 37.6±0.4 & 0±0 
10 mg-100 mg/ml & 51.3±1.9 & 12.8±1.5 & 2.1±0.6 & 32.4±2.6 & 1.5±0.2 
100 mg-1 g/ml & 57.8±0.7 & 15.5±0.6 & 1.1±0.2 & 20.9±0.8 & 4.6±0.8 
500 mg-5 mg/ml & 50.4±1.1 & 12.3±1.6 & 2.1±0.3 & 32.1±2.0 & 2.8±0.5 

Mixed monolayer of OEG-COOH/OEG thiols was created by coadsorption. The relative concentrations of OEG-COOH/OEG in solution may not be the same as their concentration on the surface, since the kinetic parameter of SAM formation depends on the molecular weight and also on their chemical properties such as charge, and hydrophobicity. The results of XPS studies on mixed monolayer surfaces are presented in Table 5. The SAM of OEG thiol is a well studied and characterized system. Harder et al reported a cross section area of 21.3 Å²/molecule [46]. The density of OEG-COOH SAM was calculated from the thickness of the SAMs as described in Chapter 2 based on the attenuation of substrate signal. The cross-sectional area of OEG-COOH molecules in the SAM was found to be 28.8 Å²/molecule. The higher cross-sectional area for OEG-COOH when compared to OEG could be due to the presence of the terminal carboxyl group that might sterically hinder the packing compared to the OEG SAM. Using the cross-sectional area for 100% OEG-COOH SAM, the COOH groups/cm² was calculated to be $3.5 \times 10^{14}$/ cm², this assumption is valid since each OEG-COOH molecule terminates with a carboxyl group. Since the OEG SAM does not possess any COOH groups we assumed the carboxyl surface concentration for OEG SAM to be zero. Figure 3 shows a plot of ratio of peak areas COOH/alkane against the surface concentration of carboxyls. The peak areas of COOH and alkane were obtained from high resolution C1s spectra shown in Figure 4. The variation of surface COOH concentration was assumed to vary linearly with change in relative proportions of OEG and OEG-COOH thiols on the surface. An estimate of the surface concentration of COOH groups present in mixed monolayers created using 25% and 75% OEG-COOH thiol
(in solution) was made by correlating the COOH/alkane ratios to the surface COOH concentration in the plot shown in Figure 3.

**Table 5.** Results of XPS studies on mixed monolayers of OEG/OEG-COOH thiols created by co-adsorption. The relative peak area percentages were calculated from the individual peaks in C 1s high resolution spectra.

<table>
<thead>
<tr>
<th>OEG-COOH % (in solution)</th>
<th>Alkane 285 eV %</th>
<th>PEG 286.8 eV %</th>
<th>COOH 289.7 eV %</th>
<th>COOH/Alkane ratio</th>
<th>COOH/cm$^2$ (on surface)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM-OEG 100%</td>
<td>50.5±0.1</td>
<td>41.1±0.1</td>
<td>4.7±0.1</td>
<td>0.092±0.002</td>
<td>3.5E+15</td>
</tr>
<tr>
<td>CM-OEG 75%</td>
<td>50.5±0.1</td>
<td>41.5±0.1</td>
<td>4.1±0.1</td>
<td>0.080±0.001</td>
<td>2.70E+15</td>
</tr>
<tr>
<td>CM-OEG 25%</td>
<td>50.8±0.1</td>
<td>42.4±0.1</td>
<td>3.6±0.1</td>
<td>0.070±0.001</td>
<td>2.00E+15</td>
</tr>
<tr>
<td>CM-OEG 0%</td>
<td>48.5±0.3</td>
<td>46.5±0.7</td>
<td>1.9±0.1</td>
<td>0.038±0.002</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 3.** A plot of COOH/alkane peak percentage ratio (y axis) against surface COOH concentration (x axis). The data points denoted by ♦ and △ correspond to OEG SAM and OEG-COOH SAM, respectively; while × and ■ represent the mixed monolayer created using 75% and 25% OEG-COOH, respectively.
ii. Determination of the enzyme content and activity

The quantification of surface subtilisin concentration was done using XPS based on the relative atomic percentage of nitrogen. Based on the calculations described in Chapter 2, the thickness of the immobilized subtilisin layer was determined. Using the value of molecular weight and assuming dry protein density of 1.27 g/cm³ [201], number of moles of subtilisin and thus the number of molecules per unit area was calculated. The results of these calculations are summarized in Figure 5. The surface concentration of subtilisin was found to increase with an increase in the surface concentration of surface COOH groups, although the increase was not linear. The enzyme concentration on surfaces with 75% and 100% OEG-COOH did not differ significantly even though their surface COOH concentration differed by 8 ×10¹⁴/cm². This suggests that there is limitation in the number of molecules of subtilisin that
can be immobilized per unit area irrespective of the number of binding sites available for covalent coupling. The surface with 0% OEG-COOH did not have any detectable amount of enzyme, indicating that there is minimum amount of non-specific adsorption. The possibility of non-specific adsorption on surfaces with significant surface concentrations of carboxyl cannot be ruled out, since at pH 7.4 (reaction buffer PBS), the carboxylic acid group carries negative charge while the subtilisin itself would be positively charged (pI 9.4), thus creating room for electrostatic interactions. The washing step should ideally remove weakly bound enzyme molecules, thus ensuring minimum non-specific adsorption.

**Figure 5.** Concentration of subtilisin on mixed monolayers of OEG/OEG-COOH, determined based on the relative atomic percentage measured using XPS. The percentage of OEG-COOH indicated on the x axis is the concentration in solution during coadsorption.

The activity of subtilisin immobilized surfaces was measured using the colorimetric assay as described in methods section. The results of colorimetric studies are summarized in Figure 6a. Since the number of enzyme molecules per unit area was already determined, it was possible to calculate the activity per molecule of subtilisin and the results of this analysis is shown in Figure 6b. The mixed OEG-COOH/OEG thiol surface with 50% OEG-COOH had an enzyme activity of approximately 455 mU/cm² with \( 4.4 \times 10^{11} \) molecules/cm², while the surface with 100% OEG COOH had much lower activity 121 mU/cm² in spite of higher subtilisin \( 8.8 \times 10^{11} \) molecules/cm². The surfaces with 0% and 25% OEG-COOH showed no
enzyme activity and also had lower enzyme concentrations when compared to higher OEG-COOH surface contents. This absence of direct correlation between enzyme concentration and surface activity could be due to autolysis of enzyme molecules, which is a well observed process with subtilisin [183] and its variants.

**Figure 6.** Enzyme activity results of mixed monolayers of OEG/OEG-COOH immobilized with subtilisin. (a) Shows the activity per unit area of surfaces expressed in mU/cm². (b) Summarizes the activity per enzyme molecule values of various surfaces. The percentage of OEG-COOH indicated on the x axis is the concentration in solution during coadsorption.

One would expect higher autolysis when the enzyme molecules are present at higher concentrations on the surface, and the nitrogen quantification indicated that 100% and 75%
OEG-COOH have higher enzyme contents when compared to 50% OEG-COOH surfaces. An alternate explanation of lower activity at high surface concentrations could be due to restrictions on activity due to higher packing, which might hinder the accessibility of the active site of the enzyme towards the substrate. Apart from autolysis, denaturation is also a significant process that hinders the success of creating subtilisin immobilized surfaces with optimum activity. Several groups working with subtilisin solved the denaturation issue by protein engineering techniques to enhance the stability of naturally occurring enzyme molecules [178, 179].

iii. Bacterial adhesion studies

The results of bacterial adhesion studies on subtilisin immobilized mixed SAMs of OEG-COOH/OEG thiol are presented in Figure 7. The surfaces with 50% and 100% OEG-COOH thiol had approximately the same number of cells, but the 75% OEG-COOH surface had a cell count approximately higher by a factor of 5. The order of increasing enzyme activity of surfaces is 0% = 25% < 100% < 75% < 50%. There was no correlation between the results of enzyme activity studies and bacterial adhesion assays. Also, the amount of enzyme on the surfaces did not correspond either to the enzyme activity on the surfaces or to the number of bacteria adhering to the surfaces. This could be due to the fact that subtilisin is incapable of minimizing the adhesion of S. xylosus. This absence of direct correlation between enzyme concentration and surface activity could be due to autolysis of enzyme molecules which is well known process observed with subtilisin [183]. The subtilisin immobilization studies on mixed monolayers of OEG/OEG-COOH thiols helped in understanding the relationship between the surface coverage and enzyme activity. With this understanding further investigations were done by performing enzyme immobilization studies on mixed layer of PEG-COOH and OEG thiol. The bacterial inhibition ability of subtilisin was tested using active and denatured forms of the enzyme immobilized on PEG-COOH surfaces backfilled with OEG thiol. The preparation of these surfaces was discussed in Chapter 3. The denatured subtilisin molecules do not possess proteolytic activity and thus if proteolytic activity is incapable of preventing bacterial adhesion, then there should not be any significant difference in bacterial adhesion on surfaces functionalized with active and denatured enzymes. Also, S. xylosus
could be outside the list of bacterial strains that can be prevented from forming biofilms by subtilisin. *S. xylosus* was initially chosen since it is highly relevant to the dairy industry. It has been reported that *S. aureus* can be prevented from forming biofilm by subtilisin like proteases [202] and hence the subtilisin based surfaces were tested against *S. aureus*. The motive behind this study is to understand the effect of backfilling on the performance of immobilized enzyme.

**Figure 7.** Results of bacterial adhesion assays performed using *S. xylosus*, OD-0.01, incubation time 2h, 37 °C (y-axis: cells/μm²). The bacterial count on gold was 1.7±0.2×10⁻³

**B. Subtilisin immobilization on mixed PEG-COOH/OEG surface**

**i. XPS characterization of backfilled PEG-COOH surface**

The preparation and characterization of surfaces formed by backfilling PEG-COOH SAM with OEG or R thiol was discussed in detail in chapter 3. Some of the important results of the XPS analysis are summarized in Table 6. Since the ratio of the alkane/ether peak area for OEG SAM is known, it is possible to estimate the relative contribution made to the PEG peak from OEG thiol molecules present in the backfilled PEG-COOH SAM using the area of the alkane peak. Once the contribution of OEG thiol is deducted, it is possible to estimate the thickness of the PEG-COOH overlayer, and using the thickness the graft density can be calculated, as described in chapter 2. Since each PEG-COOH molecule is end capped with one carboxyl group, and OEG thiol or R has no carboxyl groups, thus it can be assumed that the number of PEG-COOH molecules per unit area is same as the number of carboxyl groups.
per unit area. The results for the carboxyl concentration estimations on backfilled PEG-COOH surfaces are summarized in Figure 8.

**Table 6.** Summarizes results of XPS analysis of PEG-COOH SAM backfilled with OEG or R thiol molecules. The Au%, O/C and S% were obtained from survey spectra. The alkane/PEG ratio and COOH% were obtained from high resolution C1s spectra.

<table>
<thead>
<tr>
<th></th>
<th>PEG-COOH</th>
<th>PEG-COOH+OEG</th>
<th>PEG-COOH+R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au %</td>
<td>39.0± 0.4</td>
<td>36.2± 0.5</td>
<td>41.6± 0.5</td>
</tr>
<tr>
<td>O/C</td>
<td>0.49±0.03</td>
<td>0.42±0.01</td>
<td>0.40±0.01</td>
</tr>
<tr>
<td>Alkane/PEG</td>
<td>0.1± 0</td>
<td>0.3± 0</td>
<td>0.4± 0</td>
</tr>
<tr>
<td>S %</td>
<td>2.1± 0.2</td>
<td>1.9± 0.6</td>
<td>2.1± 0.2</td>
</tr>
<tr>
<td>COOH %</td>
<td>4.5± 0.3</td>
<td>3.5± 0.1</td>
<td>3.6± 0.3</td>
</tr>
</tbody>
</table>

In Chapter 3 it was speculated that backfilling PEG-COOH SAM with R thiol molecules results in a higher amount of PEG-COOH desorption when compared to that caused by OEG thiol addition. Desorption of PEG-COOH molecules should cause a decrease in the surface concentration of carboxyl groups. The results of our estimation of COOH concentration clearly shows that PEG-COOH surface backfilled with R thiol has approximately 20% lower COOH concentration than that of the PEG-COOH SAM or the SAM backfilled with OEG thiols as shown in Figure 8. In Chapter 3 it was demonstrated that backfilling PEG-COOH SAM using OEG thiols improved its ability to resist serum adsorption. Based on these results we expect minimum non-specific adsorption of subtilisin on PEG-COOH+OEG when compared to PEG-COOH SAM or PEG-COOH+R.
ii. Subtilisin immobilization on mixed PEG-COOH SAMs

Subtilisin enzyme was immobilized on mixed monolayers of PEG-COOH SAM backfilled with OEG or R thiols as described in the methods section. XPS was used to quantify the amount of enzyme immobilized on PEG-COOH based SAMs. The relative atomic percentage was used to determine the thickness of immobilized enzyme layer, using this value the enzyme molecules per unit area was estimated as described in the previous section. The surface concentrations of enzyme on PEG-COOH SAM, PEG-COOH+OEG and PEG-COOH+R are summarized in Figure 9.
Surprisingly, there was no significant difference in the amount of enzyme on surface between the three surfaces analyzed. The enzyme immobilized surfaces did not differ in their colorimetric activity values and also did not exhibit any significant difference in activity per enzyme molecule value as shown Figure 10 and Figure 11, respectively.

**Figure 10.** Colorimetric activity of subtilisin immobilized surfaces; PEG-COOH SAM, PEG-COOH+OEG and PEG-COOH+R. Activity unit: mU/cm².

**Figure 11.** Activity per subtilisin molecule immobilized on a PEG-COOH SAM backfilled with OEG and R thiol. The estimation was based on N% obtained from XPS survey spectra. Activity unit: mU/molecule.
iii. Bacterial adhesion studies on subtilisin immobilized mixed PEG-COOH/OEG surfaces

Bacterial adhesion studies using *S. aureus* were performed on mixed monolayers immobilized with active and denatured enzyme using the procedure described in methods section. The inclusion of denatured enzyme would help to check the capability of subtilisin to minimize bacterial adhesion. The results of adhesion studies are shown in Figure 12. The subtilisin immobilized surfaces resist bacterial adhesion better than the surfaces immobilized with denatured subtilisin. For example, the SAM of PEG-COOH with active subtilisin has a cell count of $11\times10^{-3}$ cells $/\mu m^2$, whereas with denatured subtilisin a much higher cell count of $56 \times10^{-3}$ cells $/\mu m^2$ was observed. This result confirms there is some activity of subtilisin is capable of decreasing the adhesion of *S. aureus*. Also, the PEG+OEG surface with no enzyme is the best among all surfaces in resisting bacterial adhesion. Thus, we conclude that native subtilisin A immobilized on PEG-COOH or OEG-COOH is not any better than just the PEG-COOH or OEG thiol SAMs at resisting bacterial adhesion, despite the detection of significant amount of enzyme activity.

![Figure 12: Bacterial adhesion assay results performed using S. aureus, OD-0.1, incubation time 5h, 37°C (y-axis: cells/$\mu m^2$) on PEG, PEG+R, PEG+OEG surface; bare PEG-COOH mixed surfaces (NoEnz), active enzyme (ActEnz) and denatured enzyme (DeEnz) immobilized surfaces. Coupling was done by using sulfo-NHS-EDC catalyzed reaction as described in methods section. The count on alkane thiol (R) SAM was 11±0.4](image-url)
Therefore, the subtilisin immobilized surface may not be efficient enough to be an effective antifouling surface in comparison to the PEG-only surfaces. The average N% obtained using PEG-COOH based surfaces is higher than OEG-COOH based surfaces. Although the N% of the PEG-COOH or PEG-COOH+OEG is higher, the activity measured using the colorimetric assay is lower than that for OEG-COOH (50%, 75% and 100%) by at least a factor of two. This much lower value could be due to a higher proportion of autolysed subtilisin molecules (as a result of high concentration) or higher proportion of denatured molecules. An alternate reason could be blocking of the active site of subtilisin enzyme at high surface concentrations. Further investigation needs to be done to find out the exact mechanism by which the subtilisin molecules lose activity upon immobilization at high surface concentrations. It was surprising to find out that the surface concentration of subtilisin reached a maximum with a NHS-EDC concentration of 100 mg-1 g/ml, and the concentration decreases at higher concentration of NHS-EDC. At higher NHS-EDC concentrations one might expect higher surface concentrations due to availability of greater number of activated carboxyl groups for amide bond formation with amine residues of the enzyme. On the contrary, the opposite effect is observed with subtilisin. In literature examples can be found of similar protein immobilization experiments made using NHS-EDC concentrations higher than 100 mg-1 g/ml by a factor of 10 at least [194, 199]. The main difference between those systems and the here was the surface of immobilization and the protein being immobilized. To rule out the possibility that the backfilled PEG-COOH surface could be responsible for the observation of lower protein coupling at high NHS-EDC concentrations, immobilization experiments were performed on mixed PEG-COOH monolayers using lysozyme and BSA. Lysozyme was chosen since its pI is closer to that of subtilisin. More importantly lysozyme is a glycoside hydrolase capable of damaging the integrity of extracellular polysaccharides of bacterial cells and could potentially prevent them from adhering to surfaces. Also, the enzyme has been shown to be a robust protein capable of being immobilized on various types of surfaces such as steel, titania glass and range of organic polymers with significant retention of activity [172, 203, 204]. BSAs is commonly used in most post-immobilization steps to block the sites of non-specific adsorption.
C. Immobilization of Lysozyme and BSA

Immobilization experiments with lysozyme and BSA were performed in order to find out if the PEG-COOH mixed surface created by backfilling with OEG thiols is capable of minimizing non-specific adsorption during covalent coupling of proteins. Immobilization was identical to those described. The results of nitrogen quantification using XPS are shown in Figure 13.

![Figure 13. Studies on lysozyme and BSA immobilized SAMs of PEG and PEG+OEG (PEG-COOH thiol SAM backfilled with OEG thiols). Protein molecules immobilized with and without the use of EDC/NHS. The y-axis shows the % of N measured using the survey spectra of XPS.](image)

In order to gain insight into the amount of non-specifically adsorbed protein, experiments were carried out with and without EDC/NHS. In the absence of EDC/NHS no covalent coupling of Lysozyme and BSA can occur. It was discussed in Chapter 3 that the PEG-COOH+OEG surface is better at preventing non-specific adsorption when compared to PEG-COOH SAMs. From the XPS results, nitrogen signals were absent on PEG-COOH+OEG surfaces exposed to BSA and lysozyme without EDC/NHS, whereas 3.8% N and 1.3% N were detected on the surfaces of PEG-COOH SAMs, respectively. It should be noted that we observed similar results ( 0% N ) during immobilization experiments performed using subtilisin without NHS-EDC on mixed OEG-COOH/OEG monolayers created by co-adsorption (data not shown). In the experiments using EDC/NHS activation, the N% on PEG-
COOH and PEG-COOH+OEG was 5.3% and 3.5% for BSA, and 3.9% and 2.6% for lysozyme, respectively. It is speculated that the higher atomic N% with PEG SAMs in comparison to PEG+OEG is due to a non-specific protein adsorption component in addition to the contribution from covalent coupling. Thus the mixed PEG-COOH+OEG surface is a suitable platform for immobilizing active biomolecules using the conventional protocols available in the literature that use higher NHS-EDC concentrations than the optimal concentration required for immobilization subtilisin.

IV. Conclusions and discussion

Immobilized subtilisin was investigated on co-adsorbed OEG-COOH/OEG surfaces using an enzyme activity assay, nitrogen quantification using XPS and bacterial adhesion assays with *S. xylosus*. It was found that a large proportion of immobilized subtilisin molecules were inactive. There was no correlation between the amount of enzyme on surface and the number of bacterial cells adhering onto it. This could be possible if the enzyme molecules denature or autolyse upon immobilization at high surface concentrations. Immobilization studies using active and denatured subtilisin molecules on PEG-COOH surface backfilled with OEG thiol showed that the active enzyme activity is indeed capable of reducing the adhesion of *S. aureus* relative to denatured enzyme. The bare PEG-COOH and PEG-COOH+OEG surface were better at resisting bacterial adhesion (*S. aureus*) than subtilisin immobilized surfaces. Since the carboxyl activation conditions were different for PEG-COOH and OEG-COOH surfaces, direct comparisons could not be made for results of enzyme activity, N atomic percentage and bacterial adhesion studies. Immobilization studies using lysozyme and BSA showed that PEG-COOH+OEG surfaces have minimum non-specifically adsorbed protein. Control experiments without the NHS-EDC activating agent demonstrated that PEG+OEG surfaces are capable of efficiently resisting non-specific adsorption and hence most of the adsorbed protein is covalently coupled. The bacterial attachment studies using *S. xylosus* and *S. aureus* on subtilisin immobilized surface show that the attachment of former bacterial species is unaffected by the activity of enzyme under the conditions used in this work, while the attachment of later seem to be minimized by activity of enzyme. This difference in the effect of enzyme on attachment could be due to the difference in the biological mechanism.
used by them in the initial adhesion or structural differences in the extracellular matrix surrounding the cell membrane. It should be noted that in the experiments with denatured enzyme it was assumed that the surface concentration is same as that of obtained by immobilization of active enzyme. The quantitative difference in the bacterial attachment between surfaces immobilized with active and denatured enzyme could be due to difference in amounts of protein immobilized rather than the activity of enzyme. Hence the confirmation of role of enzyme activity in minimizing attachment of *S. aureus* further quantification of surface immobilized with denatured enzyme.
CHAPTER 5
Grafting of poly(ethylene glycol) (PEG) layers using supercritical carbon dioxide

I. Introduction

In our daily lives we commonly encounter matter in three phases; gas, liquid and solid. Although the needs for most of our applications can be met with these phases of matter, alternate phases such as plasma and supercritical fluids possess unique characteristics. When a gas is compressed by increasing pressure or lowering temperature, it undergoes a phase transition from gas to liquid and finally to solid. But under a certain range of pressure-temperature conditions this phase transition results in a supercritical fluid (SFC) (Figure 1a). SFC has a solvation ability close to that of a liquid while its diffusivity is similar to that of a gas (Table 1). Thus the use of SFC as a solvent combines the advantages of liquids and gases [205-207]. SFCs have extremely low surface tension, and thus high wettability. These unique physical properties make the use of SFC very popular in small and large scale industrial applications such as extraction or purification of natural products, reaction media, polymer processing and nanoparticle synthesis [205, 207, 208]. Supercritical carbon dioxide (scCO$_2$) is the most used SFC since it is a ‘green’ solvent and enters the supercritical phase at relatively moderate temperature-pressure conditions of 31°C/7.3 MPa [206] when compared to other commonly used SFCs such as water and methanol. Several systematic studies have been conducted to understand the relationship between the partition coefficient of molecules in SFC and various pressure-temperature conditions [209]. In general, with an increase in pressure, the solubility increases because of increases in solvent density [208-212]. The temperature generally is inversely related to solubility (also with solvent density) [208], but this relationship can be directly proportional instead of inverse if the vapor pressure of a compound being dissolved is significantly high [210]. Apart from studying small molecules, studies have been conducted extensively on the interactions of macromolecules such as polymers within SFCs. Some of the well studied polymers are polyethylene, polystyrene, poly(methyl methacrylate) and other industrially relevant polymers [211-214]. The solubility of
polymers in SFC depends on the pressure and temperature similar to the solubility of small molecules [211, 212].

**Figure 1:** (a) The phase diagram of CO$_2$ at various ranges of pressure and temperature, including the supercritical range. (b) A typical set-up used for compressing and heating CO$_2$ gas to make it supercritical.

**Table 1:** Comparison of the physical properties of solvent in gas, supercritical and liquid phases [205].

<table>
<thead>
<tr>
<th></th>
<th>Density (kg/m$^3$)</th>
<th>Viscosity (mPa.s)</th>
<th>Diffusivity (mm$^2$/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas</td>
<td>1</td>
<td>10</td>
<td>1-10</td>
</tr>
<tr>
<td>SFC</td>
<td>100 - 1000</td>
<td>50 - 100</td>
<td>0.01 – 0.1</td>
</tr>
<tr>
<td>Liquid</td>
<td>1000</td>
<td>500 - 1000</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The addition of co-solvents such as ethanol, water and toluene to scCO$_2$ alters the solubility of polymers [211]. Co-solvents increase the polarity of scCO$_2$ thus improving its ability to solubilize polar macromolecules. There are many important parameters that influence the solubility of polymers in SFC such as molecular weight, polarity, type of end groups and molecular architecture (e.g. branched/linear). As already discussed in Chapter 1, for applications involving grafting polymers onto surfaces, the graft density depends on the solubility of a polymer in a given solvent. The physical properties of SFC can be easily manipulated thus it can be a versatile solvent in the field of surface science. Applications
requiring solid phase chemical modification (reaction of bulk rather than just the surface) of polymeric materials are efficiently done by scCO$_2$ since it has the ability to diffuse into polymeric materials (also known as swelling). For example, Liu et al. [213] reported solid state grafting of maleic anhydride onto polypropylene using scCO$_2$, and Kunita et al. [214] grafted glycidyl methacrylate onto polypropylene surfaces using scCO$_2$. Both Liu et al. and Kunita et al. demonstrated that scCO$_2$ is an efficient solvent for solid state transfer of reactants onto polypropylene. SFCs such as scCO$_2$ have the unique ability to chemically modify buried interfaces. Jia et al. used scCO$_2$ to perform a silane based surface functionalization of silanol groups at the silicon/polystyrene interface (silanol groups buried underneath the polystyrene layer) [215]. They showed that the supercritical CO$_2$ phase was the reason behind the success of the modification that could not be achieved using a liquid solvent. There have been a few other reports on silane based functionalization of alumina [216], titania [216] and silica [217] surfaces using scCO$_2$. Although there are a number of reports on scCO$_2$ based surface modifications, there are no reports on grafting of polymeric chains onto surfaces. In this chapter results are presented involving grafting of PEG thiol molecules onto gold substrates using scCO$_2$.

One of the main strategies used in ‘grafting to’ techniques is the use of ‘cloud point’ grafting, where the solubility of the polymer (e.g. PEG) is reduced using high ionic strength and high temperature, thereby decreasing the excluded volume effects leading to high graft densities. Cloud point conditions are easily attained using SFC by systematic variation of the pressure-temperature conditions. There have been some reports on the effect of molecular weight, end groups, pressure, temperature and co-solvent on the partition coefficients of PEG in scCO$_2$ [212, 218, 219]. There are no reports on studies involving effects of pressure-temperature conditions on the surface PEG graft density. This chapter presents the results of studies on grafting PEG thiol on gold using scCO$_2$ at different conditions of pressure-temperature and co-solvents. Comparisons of these results are made using conventional liquid solvents such as ethanol, toluene and methanol.
II. Materials and methods

A. Chemicals

α-methoxy-ω-thiol poly(ethylene glycol) (2, 5 or 10 KDa PEG thiol, >98% purity) were purchased from Laysan Bio Inc. (Alabama, USA), Lysozyme (Lyz), BSA, Casein, 25% ammonium hydroxide, 30% hydrogen peroxide, absolute ethanol (EtOH), methanol (MeOH) and toluene (Tol) were purchased from Sigma Aldrich (Aarhus, Denmark). Ultrapure MilliQ (MQ) water with a resistivity of 18.2 MΩ was used for making ethanolic solutions.

B. Substrate preparation and functionalization

Gold substrates were prepared by sputtering 50 nm gold layer onto a 3 nm titanium adhesion layer on silicon wafers in the same way as mentioned in Chapter 3. The gold surfaces were cleaned by UV/ozone treatment for 30 minutes, followed by treatment with basic piranha solution (H₂O: NH₄OH: H₂O₂ in ratio 4:1:1) at 70°-80°C for 5 minutes and then rinsed with MQ water. (CAUTION: Piranha solution reacts violently upon contact with organic solutions). Grafting in liquid unless otherwise mentioned was done by immersing gold slides for 3h at room temperature and ambient pressure (RTP 20°C/101 kPa) in 0.2 mM PEG thiol solution, followed by rinsing with MQ water to remove any physically adsorbed molecules. The SFC based grafting unless otherwise mentioned was done using a set-up shown in Figure 1b. The gold slides and appropriate amount of PEG thiol were placed in the SFC chamber (30 ml capacity), followed by filling up with CO₂ and adjustment to the required pressure-temperature. Experiments with scCO₂-co-solvent (5% co-solvent v/v) mixtures were performed by dissolving PEG thiol in 1.5 ml liquid co-solvent, which was placed in the SFC chamber followed by placing of the gold slides. Precaution was taken to prevent the gold slides coming into contact with the liquid by placing them on a Teflon support to keep the substrate holder above the liquid at the bottom of SFC chamber. Unless otherwise mentioned the SFC based functionalization was performed for 30 minutes at the required pressure-temperature condition. Once the SFC chamber was depressurized, the slides were rinsed with MQ to remove any physically bound PEG thiol molecules.
C. X-ray photoelectron spectroscopy

XPS spectra were recorded the same as mentioned in Chapter 3. Overlayer thicknesses were calculated based on attenuation of the substrate (Au) signal using Equation 8, as described in Chapter 2 in the XPS section.

\[ z = \lambda \cos \theta \ln \left( \frac{I}{I_0} \right) \quad \text{Eqn (8)} \]

Where \( z \) - overlayer thickness, \( \lambda \) - attenuation length, \( \theta \) is take-off angle, \( I \) is substrate signal with a thin film coating, \( I_0 \) bare substrate intensity.

The quantification of protein adsorption was done using the relative atomic percentage of nitrogen calculated based on the N 1s peak obtained from survey spectra of surfaces exposed to 1 mg/ml lysozyme (Lyz) or bovine serum albumin (BSA) or casein in PBS at 37°C for 24h. The protein exposed slides were rinsed with PBS followed by rinsing with MQ and dried with nitrogen gas.

D. Ellipsometry

The overlayer thickness of PEG thiol monolayers were measured using ellipsometry. An ELX-02C (DRE, Germany) ellipsometer with a He-Ne laser (632.8 nm) was used with an incident angle of 70°. The calculations were performed according to the methods of Unsworth et al. [150]. For the PEG modified surfaces, the polarizer (P) and analyzer (A) angles for the null condition were measured and thickness values were determined using a three-layer (air–PEO–gold) model. Complex refractive-index values \((n_i + i\kappa_i)\) of 0 + i0 and \((n_f + i\kappa_f)\) of 1.47 + i0 were used for air and the PEG films, respectively. Final thickness values were obtained by fitting the experimental data to the model using the Ellips 32 software.

E. Quartz crystal microbalance with dissipation (QCM-D)

QCM-D studies were performed using a Q-Sense E4 system (Gothenburg, Sweden). The PEG thiol functionalization using either scCO₂ or liquid solvents was performed directly on QSX 301 crystals (Q-Sense) coated with 100 nm gold having an approximate resonance frequency of 5 MHz. Protein adsorption experiments using BSA, casein and Lyz were
performed in triplicate. Functionalized gold coated crystal surfaces were equilibrated at 37°C in PBS (100 mM pH 7.4) prior to exposure to the protein solutions. Protein adsorption studies were carried out using 1 mg/ml Lyz or BSA or casein in PBS (pH 7.4) at 37°C. Once a stable baseline was attained, the surfaces were exposed to 750 μL of protein solution delivered at a flow rate of 100 μL/minute followed by rinsing with PBS buffer. The shift in resonance frequency, corresponding to the 7th overtone, was used to monitor the protein adsorption.

III. Results

A. Preliminary studies

Kingshott et al. have previously shown that covalent attachment of PEG layers is very efficient in preventing fouling for a longer period of time when compared to non-covalently attached PEG molecules [102]. Hence the nature of chemical interaction between PEG and substrate is very important for determining the non-fouling efficiency of a PEG coating. Since there have been no studies on the quality of PEG monolayers grafted using scCO2, preliminary studies involved comparing the surfaces prepared from SFC and ethanol to confirm that the scCO2 based PEG thiol grafting results in a monolayer that is chemically similar to that formed from conventional solvents such as methanol. The main parameters considered are the thickness of overlayer and the relative percentages of bound to unbound thiols. The thickness was calculated using XPS, as described in Chapter 2. The high resolution S2p spectra were used to determine the percentage of bound thiol, which represents the amount of covalently bound PEG thiol molecules, as described by Castner et al. [128].

The pressure-temperature conditions of SFC grafting used in this work were chosen based on reports by Daneshwar et al. [219], Drohmann et al. [212] and Mishima et al. [218]. The preliminary SFC experiments were performed at 20 MPa/60°C and under these conditions we expect a solubility of 0.5-1.5 wt% or approximately 5 mg/ml (assuming the density of SFC to be 0.5 g/ml). Since there are no reports on the time required for PEG thiol self assembly using SFC, our preliminary studies were aimed at optimizing the time needed for monolayer formation. Figure 3 summarizes the results of kinetic studies involving PEG
thiol functionalization carried out for varying periods of time using 75% liquid ethanol (room temperature/pressure, 25°C/101 kPa) and scCO₂ (60°C/20 MPa). The PEG thiol monolayer formed using liquid ethanol after 30 minutes had approximately 40% bound S and this value increased with the increase in the incubation time. Thus grafting in liquid ethanol for several hours is required for the formation of a monolayer with an optimum amount of bound S. The highest thickness attained in 75% ethanol was measured to be approximately 5.5 nm. On the other hand, functionalization using scCO₂ for 30 min produced a layer that was 6 nm thick with approximately 63% bound S. Since the experimental set up used in scCO₂ based grafting requires a minimum time of 20 minutes to attain the set pressure/temperature condition, it was impossible to do grafting experiments with incubation time less than 30 minutes. It is possible that scCO₂ based functionalization of PEG thiol may require a much lower incubation time than 30 minutes. Based on preliminary studies we decided to carry out all our SFC based functionalization experiments for 30 minutes and liquid based self assembly for 3 hours. The faster reaction in scCO₂ could be due to faster diffusion of PEG thiol molecules since scCO₂ has a lower viscosity than liquids. Thus, it is concluded that scCO₂ requires a much lower time for forming a PEG thiol monolayer with an optimal proportion of covalently bound molecules. Therefore, faster kinetics and the high wettability of scCO₂ make it an ideal solvent for efficiently grafting polymers such as PEG onto surfaces.

\[\text{Figure 2: (a) Overlayer thickness; (b) percentage of bound sulfur of a 2 kDa PEG thiol SAM grafted using 75\% ethanol (EtOH:25°C/101 kPa), and scCO₂ (SFC:60°C/20 MPa) measured using XPS.}\]
The non-fouling abilities of the 2 kDa PEG thiol SAM coated using scCO$_2$ (SFC: 60°C/20 MPa) and 75% ethanol (3h/25°C/101 kPa) were compared by determining the nonspecific adsorption of Lyz, BSA and casein. Since this project is in collaboration with a milk producing company (Arla Foods A/S), the protein casein was included which is known to be the most abundant milk protein [220]. Lyz is an important part of immune system which provides protection against gram positive bacteria [221]; BSA is the most abundant plasma protein [222]. Protein adsorption studies were performed on the PEG thiol monolayer grafted from SFC and ethanol. These surfaces are exposed to the proteins and the amount of protein adsorbed on the surfaces was quantified using XPS and QCM. The results of protein adsorption studies are summarized in Figure 3.

**Figure 3**: Adsorption studies of BSA, Lyz and casein quantified using XPS and QCM on gold surfaces grafted with 2 kDa PEG thiol using liquid 75% ethanol (EtOH: 3h/25°C/101 kPa) and scCO$_2$ (SFC: 30min/20 MPa/60°C). Figure 3a shows relative amounts of protein

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**Protein adsorption studied using XPS (N %)**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gold</th>
<th>EtOH</th>
<th>SFC</th>
<th>Gold</th>
<th>EtOH</th>
<th>SFC</th>
<th>Gold</th>
<th>EtOH</th>
<th>SFC</th>
</tr>
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<tbody>
<tr>
<td>BSA</td>
<td>16</td>
<td>12</td>
<td>8</td>
<td>12</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Lyz</td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Casein</td>
<td>12</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

**Protein adsorption studied using QCM (Frequency shift Hz -ve)**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gold</th>
<th>EtOH</th>
<th>SFC</th>
<th>Gold</th>
<th>EtOH</th>
<th>SFC</th>
<th>Gold</th>
<th>EtOH</th>
<th>SFC</th>
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<tbody>
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<td>22.1</td>
<td>72.5</td>
<td>22.1</td>
<td>72.5</td>
<td>72.5</td>
<td>72.5</td>
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<td>72.5</td>
</tr>
<tr>
<td>Lyz</td>
<td>25</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Casein</td>
<td>25</td>
<td>15</td>
<td>15</td>
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<td>15</td>
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adsorbed after exposure to 1 mg/ml protein solution for 24 hrs on various PEG surfaces, N% on the y-axis which is directly related to amount of protein adsorbed, while the y-axis on Figure 3b shows the change in resonance frequency (-ve) of QCM crystals upon protein adsorption (1 mg/ml, 750 \( \mu l \), flow rate 100 \( \mu l/minute \)).

The QCM results show that the surface grafting of PEG thiol using ethanol and SFC results in overlayers that are equally efficient in resisting protein adsorption as shown in Figure 3b. It must be noted that the QCM studies were using a total volume of 750 \( \mu l \) of 1 mg/ml protein solution over a period of 7.5 minutes; whereas the XPS quantification studies were done on gold slides exposed to 1 mg/ml protein solutions over a period of 24h, thus the results of QCM and XPS cannot be directly correlated. The XPS results show that SFC based grafting is better at resisting protein adsorption owing to a higher PEG layer thickness and higher proportion of bound sulfur determined from S 2p spectra. Thus based on chemical characterization of PEG thiol monolayers via XPS and protein adsorption studies via QCM, it is preliminary concluded that scCO\(_2\) based PEG grafting results in a monolayer that is very similar to those produced by conventional liquid solvents such as ethanol. The QCM based short term protein adsorption studies showed no difference between PEG SAMs grafted using scCO\(_2\) and ethanol. The quantification of 24 h protein adsorption studies showed that PEG SAM grafted using scCO\(_2\) has better resistance to protein adsorption than the SAM grafted using ethanol.

**B. Grafting studies in liquids**

The effect of polymer molecular weight on the graft density of SAMs was discussed in section 1d of Chapter 1. There is an inverse relationship between graft density and molecular weight of polymer molecules. Graft density can also be varied by altering the solubility of polymer molecules in the grafting solvent. The solubilities of PEG in ethanol, toluene and water vary in the following order; water > toluene > ethanol. This order was deduced experimentally by comparing the sonication time required for dissolving 2 kDa PEG thiol in the three solvents at room temperature. Thus, by mixing ethanol with water or toluene, it is possible to systematically vary the solubility of PEG and thus the graft density. The objective was to compare the effect of solubility of PEG thiols on the surface graft density of SAMs
formed from liquid and scCO$_2$ solvents. Grafting studies in liquid were done on gold slides using various mixing ratios of ethanol and water/toluene. The overlayer thicknesses were determined using XPS and ellipsometry as described in the methods section. The results of studies using 2 kDa, 5 kDa and 10 kDa PEG thiol are shown in Figures 4a, 4b and 4c, respectively. It was observed that increasing the solubility of PEG by adding water to ethanol results in a decrease in the overlayer thickness, as the proportion of added water increases. For example the thickness of 2 kDa PEG thiol monolayer grafted using 75 % ethanol is half of what was obtained from 100 % ethanol as shown in Figure 4a. The reason for difference in values between XPS and ellipsometry will be dealt with in the discussion section. The XPS and ellipsometry measurements showed similar trends of changing overlayer thickness with varying solubility. All the thickness comparisons in this section (III b) are discussed based on ellipsometry results for the sake of simplicity. For 100% ethanol, it was observed that PEG thiol did not readily dissolve in the solvent, so the solutions had to be sonicated for 30 minutes.

**Figure 4a:** Overlayer thicknesses of 2 kDa PEG thiol SAM grafted using mixtures of ethanol(EtOH)/water(H$_2$O) and ethanol(EtOH)/toluene(Tol) (3h/25°C/101 kPa), measured using ellipsometry and XPS.
Figure 4b: Overlayer thicknesses of 10 kDa PEG thiol SAM grafted using mixtures of ethanol (EtOH)/water (H₂O) and ethanol (EtOH)/toluene (Tol) (3h/25°C/101 kPa), measured using ellipsometry and XPS.

Figure 4c: Overlayer thicknesses of 5 kDa PEG thiol SAM grafted using mixtures of ethanol (EtOH)/water (H₂O) and toluene (Tol) (3h/25°C/101 kPa), measured using ellipsometry and XPS.
The 10 kDa PEG thiol solution in 100 % ethanol continued to remain cloudy in spite of the prolonged sonication, while the 2 kDa and 5 kDa PEG temporarily dissolved during the sonication but rapidly became cloudy in 10-20 min once the sonication step was stopped. The 10 kDa PEG thiol solutions prepared using ethanol/toluene mixtures with 97.5 % or lower percentage of ethanol formed clear solutions upon sonication for 30 minutes. This suggests that grafting in 100 % ethanol occurs at cloud point conditions very similar to that reported by Kingshott et al. using high ionic strength solutions [41]. This alternative way of cloud point grafting based PEG thiol functionalization using ethanol can be extended to reactions other than Au-thiolate formation provided the reactants are soluble in and stable towards ethanol.

Since toluene is also an important solvent in polymer grafting, studies were performed on the effect of solubility of PEG in ethanol-toluene mixtures on the graft density of PEG thiol layers. The solubility of PEG thiol in toluene was lowered by the addition of ethanol. The results of overlayer thickness of 2 kDa and 10 kDa PEG thiol molecules grafted with various proportions of toluene-ethanol mixtures are given in Figure 4a and 4b, respectively. With increasing percentage of ethanol the thickness of overlayer increases. The thickness of 10 kDa PEG thiol monolayer grafted using 25% toluene solution results in a overlayer with a thickness of 1.8 nm, where as the thickness is 4.4 nm when 1% toluene is used, as shown in Figure 4b. Thus it could be concluded from the liquid based PEG thiol grafting study that decrease in solubility of PEG results in higher graft densities due to decreased excluded volume effect. The effect of Mw of PEG thiol on the graft density is clearly evident upon comparing the thicknesses of SAMs of 2 kDa, 5 kDa and 10 kDa PEG thiols grafted using 100% ethanol, which are 7.6nm, 10.9nm and 5.5nm respectively. Thus the order of the thicknesses are 5 kDa>2 kDa>10 kDa.

Since the 5 kDa PEG thiol formed SAM of greatest thickness as seen from the results presented so far in this chapter, comparative studies in scCO₂ mainly focused on 5 kDa PEG apart from the preliminary studies using 2 kDa PEG thiol. It should be noted that in this section, there was no comparison of percentages of bound sulphur unlike the previous preliminary studies section. Since the PEG thiol SAM has the sulfur atom below the layer of PEG, the S photoelectron signal attenuation would increase with increase in PEG thiol SAM.
thickness. The S signal became extremely low for samples that had thicknesses >7-8nm, peak fitting of the high resolution S 2p spectrum into bound and unbound thiol species was thus difficult due very low signal intensity. To ensure that a significant proportion of PEG thiol molecules in the SAM are chemisorbed, the samples with PEG thiol SAM thickness of >7-8nm were sonicated for 30 minutes followed by XPS and ellipsometry measurements. Sonication is known to cause desorption of loosely physisorbed molecules from SAMs. The sonication step did not result in any significant decrease in thickness (data not shown), thus confirming that these thicker SAMs have an optimal percentage of covalently bound PEG.

C. Grafting studies in scCO₂

As mentioned the solubility of a polymer can also be manipulated in SFC by altering the pressure-temperature conditions. Grafting 2 kDa and 5 kDa PEG thiols on gold was done in scCO₂ as described in section II b. The thickness of the 2 kDa PEG thiol SAM determined from ellipsometry increased from 3 nm to 4.9 nm when the temperature of scCO₂ was changed with constant pressure, from 20 MPa/60°C to 20 MPa/50°C. This increase in the thickness (Figure 5a) can be attributed to decrease in PEG solubility with decreasing temperature as described by Kirby et al. [211]. The degree of increase in the thickness was not significant when both pressure and temperature were decreased from 20 MPa/60°C to 15 MPa/50°C, and this could be due to extremely low solubility. The problem of low solubility becomes apparent from experiments with 5 kDa PEG thiol (Figure 5b), in which case a lower thickness of 2.2 nm was obtained at 20 MPa/50°C in comparison to 2 kDa PEG thiol SAM with a thickness of 4.9 nm. This decrease in thickness with increase in Mw is in agreement with de Gennes theory discussed in Chapter 1. Furthermore it can be seen that upon a decrease in temperature or pressure of the scCO₂, the thickness of the 5 kDa PEG thiol SAM does not change significantly, unlike in the case of the 2 kDa PEG thiol where the thickness increases. The low solubility issue with the 5 kDa PEG thiol or higher Mw PEG thiols could be overcome by using higher temperature-pressure conditions where the density of scCO₂ would be higher. Future experiments will need to investigate scCO₂ based 5 and 10 kDa PEG thiol grafting using pressure-temperature conditions higher than those reported in this section. Apart from using higher pressure-temperature conditions, one can also think of using co-
solvents along with scCO$_2$ to help improve the partition coefficients of high M$_W$ PEG thiol molecules. The results of studies using co-solvents are discussed in the next section.

**Figure 5a:** Overlayer thicknesses of 2 kDa PEG thiol SAM grafted using scCO$_2$, at varying pressure and temperature, measured using ellipsometry and XPS.

**Figure 5b:** Overlayer thicknesses of 5 kDa PEG thiol SAM grafted using scCO$_2$, at varying pressure and temperature, measured using ellipsometry and XPS.

### D. Grafting studies in scCO$_2$–co-solvent mixtures

As mentioned in the previous section, the solubility issues with high M$_W$ PEG thiol molecules in scCO$_2$ can be solved using co-solvents. In the literature, ethanol, toluene and methanol have been shown to increase the partition coefficients of polar molecules in scCO$_2$ [211]. The objective is to achieve optimal solubility and then gradually decrease the pressure or temperature to achieve grafting close to cloud point conditions. The main advantage of using co-solvents is that they will enable cloud point grafting at lower pressure-temperature
conditions for high $M_W$ polymers, which would be a significant cost benefit in large scale industrial applications. Figure 6b summarizes the results of 5 kDa PEG thiol SAMs grafted using scCO$_2$–5% co-solvent mixtures as described in section II b. In this section the thickness comparisons are made using XPS measurements. The effect of addition of ethanol, methanol and toluene was studied. Ethanol-scCO$_2$ at 20 MPa/60°C produced a SAM thickness (3.9 nm) that was slightly lower than that obtained using scCO$_2$ at 20 MPa/60°C (4.2 nm), but upon a decrease in temperature, the ethanol-scCO$_2$ (20 MPa/50°C) produced a thicker SAM (5.0 nm). The results of grafting experiments using 5 kDa PEG thiol with toluene-scCO$_2$ are shown in Figure 6b. Upon lowering of temperature the toluene-scCO$_2$ resulted in a SAM with lower thickness of 4.9 nm (20 MPa/50°C) when compared to 6.0 nm obtained using 20 MPa/60°C. The three solvents selected for this study were an attempt to understand the relationship between the polarity of solvent and graft density of PEG thiol SAM obtained. Based on the data obtained from XPS and ellipsometry, there is no straightforward relationship between solvent polarity and thickness. For example the increasing order of thickness obtained at 20 MPa/60°C for the three co-solvents is ethanol<toluene<methanol, whereas the increasing order of polarity is toluene<methanol<ethanol. The increasing order of solubility of PEG in liquids of co-solvents under consideration is ethanol<toluene<methanol [223], which is exactly the order obtained for thicknesses with scCO$_2$-co-solvents. Thus, the solubility of PEG in the solvents seems to play a significant role in deciding the overlayer thickness obtained in SFC-co-solvent mixtures. Based on this co-solvent study, it is conclude that the cloud point conditions can be lowered for high $M_W$ PEG thiol molecules thus enabling us to achieve higher graft densities under economical conditions. The scCO$_2$-co-solvent studies for 2 and 10 kDa PEG thiol will be made in a future project. A detailed analysis of PEG SAM thicknesses obtained for PEGs of different Mw under varying conditions of pressure-temperature and co-solvents would enable better understanding of polymeric self assembly processes in scCO$_2$. 
**Figure 6a:** Overlayer thicknesses of 2 kDa PEG thiol SAM grafted using scCO₂ with 5% co-solvent ethanol/toluene, at varying temperature, measured using ellipsometry and XPS.

**Figure 6b:** Overlayer thicknesses of 5 kDa PEG thiol SAM grafted using scCO₂ with 5% co-solvent ethanol/toluene/methanol, at varying temperature, measured using ellipsometry and XPS. ‘∗’ denotes ‘data not available’.
IV. Discussion and Conclusion

The cloud point grafting procedure reported by Kingshott et al. [41] using high ionic strength is very efficient, but cannot be applied to grafting reactions sensitive to water. Examples of types of reactions used in polymer grafting procedures were mentioned in Chapter 1. Thus, development of a simple protocol applicable to all reaction sequences is of a significant interest. Here it was demonstrated that scCO$_2$ is a very efficient solvent for grafting PEG thiol molecules, and since CO$_2$ is chemically inert towards most functional groups, it will not interfere with most industrially relevant reactions such as polymer functionalization and surface modification. From the studies presented in this chapter it can be concluded that SFC based grafting under the conditions used here produced PEG thiol SAMs of moderate graft density. For example with 2 kDa PEG thiols, the highest graft density of 1.9 chains/nm$^2$ was produced using a SFC-toluene combination. Tokumitsu et al. [165] reported a maximum graft density of 3.6 chains/nm$^2$ using 2 kDa PEG conjugated to undecane thiols self assembled on gold substrates. Dalsin et al. [166] and Pasche et al. [167] reported graft densities > 3 chains/nm$^2$ using SAMs of DOPA functionalized PEG and PLL-g-PEG respectively. Cloud point grafting is thus a very efficient technique to produce SAMs of high graft density. Since the SFC based grafting technique is in its initial stages, further research needs to done to appreciate the potentials of this simple versatile technique.

It should be noted that for all the thickness results the values obtained using XPS are consistently higher than those calculated from ellipsometry. In all the overlayer thickness estimations using XPS, an attenuation length ($\lambda$) of 3.9 nm for Au 4f photoelectrons (1404.6 eV K.E) through the PEG overlayer was used in all our calculations. The attenuation length value was based on calculations suggested by Cumpson et al. [224]. The value of $\lambda$ used in this work is close to those reported by Laibinis et al. [225] and Sofia et al. [110]. An alternate way of calculating thickness using XPS involves comparing the signal from a infinitely thick layer of PEG. As an example the thickness of PEG SAM of 5kDa PEG thiol grafted using SFC (SFC 60$^\circ$C/20Mpa/5% EtOH) was calculated using a $\lambda$ value of 2.9 nm for the O1s photoelectron [224], assuming Oxygen atomic percentage of 33.3% for an infinitely thick layer of PEG. This method of calculation gave a thickness value of 2.7 nm, while the other method
based on attenuation of gold signal gave a thickness value of 3.9 nm. The calculation based on O% signal gave values much closer to the value estimated by ellipsometry 1.7 nm. Thus calculation using O signal seems more appropriate way of estimating the thickness instead of using attenuation of gold substrate signal. All these methods of calculating \( \lambda \) assume a PEG density of 1 gm/cm\(^3\). Unsworth et al. [150] reported a PEG density of 0.8 gm/cm\(^3\) and 1.0 gm/cm\(^3\) using neutron reflectometry for 5 and 2 kDa PEG thiol SAMs grafted under cloud point conditions. Hence the PEG density could be different depending on the Mw and grafting conditions. Piehler et al. used a dry density of 1.2 gm/cm\(^3\) [226]. Since the value of \( \lambda \) depends on PEG density (\( \rho \)) [225], a wrong assumption of the \( \rho \) value would lead to an erroneous estimation of thickness. Using the equation given by Laibinis et al. a difference in density of 0.2 gm/cm\(^3\) could introduce approximately 5% error [225]. In the ellipsometry calculations, the refractive index of PEG overlayers was assumed to be 1.47 as reported by Unsworth et al. based on calculations by Tokumitsu et al. [165] (PEG is assumed to be a Cauchy layer with the two lowest Cauchy parameters \( A_n = 1.45 \) and \( B_n = 0.01 \)). The refractive index values used for ellipsometric estimation of thickness in other reports on PEG brushes are very close the value used in this Chapter. Ostaci et al. used a refractive index value of 1.45 [227], Piehler et al. used 1.5 [226] and Mehne et al. performed their calculations using 1.5 [228]. The error in refractive index values in the range of 0.05 causes a error in the calculated thickness by only few Å [125]. Although the absolute values obtained using XPS and ellipsometry were different, the trends in thickness variations under different experimental conditions hold good for both techniques.

Based on preliminary studies it was concluded that PEG thiol grafting using scCO\(_2\) results in SAMs that are similar to those formed using conventional solvents such as ethanol. It was further demonstrated that it is possible to perform ‘cloud point’ type grafting by modifying the pressure-temperature conditions of SFC. By comparing results of scCO\(_2\) based studies using 2 and 5 kDa PEG thiol molecules, it could be speculated that cloud point grafting of higher M\(_W\) PEG molecules might need higher pressure-temperature conditions when compared to that required by low M\(_W\) PEG molecules. This requirement for higher pressure-temperature can be overcome by using scCO\(_2\)/co-solvent mixtures as demonstrated from studies on 5 kDa PEG thiol SAMs grafted using methanol and toluene as co-solvents.
V. Future directions

The high wettability of SFC makes it an efficient solvent for the functionalization of extremely rough surfaces, where it is tough for conventional liquids to perform reactions due to their high surface tension. Comparisons of the PEGs grafted on rough surfaces (gold coated sand blasted steel) using SFC and conventional liquids will be made in a future project. The studies with scCO$_2$-cosolvent mixtures using 10 kDa PEG thiols are also part of a future project. Comparison of results from SFC based studies of 2 kDa, 5 kDa and 10 kDa PEG thiol SAMs would enable an understanding of the strengths and advantages of using SFC as a grafting solvent.
CHAPTER 6: Thesis summary

The work presented in this thesis mainly focused on investigating novel ‘grafting to’ strategies for self assembly of poly(ethylene glycol) monolayers intended to serve as antifouling surfaces. Extensive studies were carried out using surface sensitive techniques including x-ray photoelectron spectroscopy, ellipsometry, contact angle measurements and atomic force microscopy. Once the surfaces were characterized, their antifouling abilities were assessed either by single protein adsorption studies or serum adsorption study and also bacterial attachment assays.

The graft density of 2kDa PEG SAM can be increased by backfilling the monolayer using shorter oligo(ethylene glycol) molecules. This strategy was systematically studied using carboxyl capped PEG-COOH thiols and hydroxy-terminated tri(ethylene glycol) undecanethiol (OEG). The backfilling process was found to result in the formation of homogeneous mixed monolayers rather than islands of phase separated PEG-COOH and OEG thiol molecules. The XPS and contact angle based quantitative assessment of desorption of PEG-COOH thiol molecules from the PEG-COOH SAM during the backfilling process indicated that there was minimum desorption during addition of OEG thiol whereas an alkanethiol caused significant desorption. The QCM and XPS based serum adsorption studies using 10% FBS showed that backfilled PEG-COOH SAM was better than non backfilled PEG-COOH SAM at resisting non specific adsorption. Bacterial attachment studies using *Staphylococcus aureus* demonstrated the greater ability of backfilled surface to resist initial attachment of cells when compared to non backfilled surfaces. The OEG SAM was better than backfilled PEG-COOH SAMs in resisting both serum adsorption and bacterial attachment. The antifouling properties of PEG-COOH SAM could be even lower in the absence of carboxyl tail group which contributes a negative charge. The presence of carboxyl groups enables the immobilization of bioactive molecules such as enzymes and with backfilled OEG thiol molecules, the PEG-COOH SAM would have maximum activity and minimal non specific adsorption.

The next strategy involved the use of PEG-COOH SAM immobilized with subtilisin a
known antifouling enzyme as non-fouling surface. The covalent immobilization was carried out using an NHS-EDC catalyzed reaction between amine residues of enzyme and the carboxylic group of PEG SAM resulting in amide bond formation. Subtilisin was immobilized on mixed OEG and OEG-COOH SAMs created by co-adsorption from solutions containing varying proportions of the two different thiol molecules. The quantification of immobilized subtilisin was done based on the N atomic percentage and the activity was measured using a colorimetric assay. The surface concentration of enzyme increased with an increase in surface concentration of carboxyl groups. It was found that a large proportion of immobilized subtilisin molecules were inactive and there was no linear relationship between the surface enzyme concentration and enzyme activity. The antifouling ability of the enzyme subtilisin immobilized on co-adsorbed OEG-COOH/OEG surfaces was quantified using bacterial attachment assays with \textit{Staphylococcus Xylosus} and it was observed that there was no correlation between the amount of enzyme on surface and the number of bacterial cells adhering onto it. Immobilization studies using active and denatured subtilisin molecules on PEG-COOH surface backfilled with OEG thiol confirmed the ability of active enzymes to minimize adhesion of \textit{Staphylococcus aureus}. There was no quantitative difference in the amount of enzyme immobilized on the backfilled PEG-COOH SAM and the non backfilled SAM, which did not agree with the serum adsorption studies on the same surfaces. The main reason behind the discrepancies with regards to subtilisin immobilized surfaces could be due to the autolysis of enzyme molecules upon immobilization at high coverage. The enzyme molecules could interfere with the activity of neighboring molecules when located at close proximity. Immobilization studies using lysozyme and BSA showed that PEG-COOH SAM after backfilling with OEG had minimum non-specifically adsorbed protein. Control experiments without the NHS-EDC activating agent demonstrated that OEG backfilled PEH-COOH SAMs are capable of efficiently resisting non-specific adsorption and hence most of the attached protein is covalently coupled.

The third strategy investigated involved use of supercritical carbon dioxide to graft PEG thiol molecules under solubility conditions close to ‘cloud point’, thereby producing SAMs of high graft density due to minimized exclusion volume effects. The thickness of SAMs formed in SFC and conventional liquid solvents such as ethanol, ethanol and toluene were compared
using XPS and ellipsometry. The kinetics studies of SAM formation by 2 kDa PEG thiol in SFC and 75% ethanol indicated that the SAMs with maximum thickness and bound sulfur percentage were formed within 30 minutes in SFC, whereas 75 % ethanol required 3-6 h. QCM based adsorption studies of BSA, Lyz and casein demonstrated that both SFC and ethanol based SAMs were equally good at resisting protein adsorption. Longer studies involving protein exposure for 24h and quantifications using XPS showed that SFC based SAMs were better at resisting protein adsorption. It was also demonstrated that ‘cloud point’ type grafting can be performed in SFC by modifying the pressure-temperature conditions. By comparing results of scCO$_2$ based studies using 2 and 5 kDa PEG thiol molecules, it can be speculated that ‘cloud point’ grafting of higher $M_W$ PEG molecules might need higher pressure-temperature conditions when compared to that required by low $M_W$ PEG molecules. This requirement for higher pressure-temperature can be overcome by using scCO$_2$/co-solvent mixtures as demonstrated from studies on 5 kDa PEG thiol SAMs grafted using methanol and toluene as co-solvents. Thus, this work demonstrated that SFC has the potential to be suitable solvent for grafting non-fouling polymers and when combined with suitable co-solvents, it is capable of serving as a versatile solvent to carryout surface modifications relevant to the designing of successful material surfaces that control the interactions with biological environments.
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198. CHROMOGENIC SUBSTRATES UNIVERSITY


Appendix

Mixed poly (ethylene glycol) (PEG) and oligo (ethylene glycol) (OEG) layers on gold as non-fouling surfaces created by backfilling

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ABSTRACT

Backfilling a self-assembled monolayer (SAM) of long PEG with short PEG is a well known strategy to improve its non-fouling potential. Here we show, using x-ray photoelectron spectroscopy (XPS), contact angle and atomic force microscopy (AFM) that backfilling PEG thiol monolayer with OEG terminated alkane thiol molecules results in underbrush formation. We also confirm the absence of phase separated arrangement which is commonly observed with backfilling experiments involving SAM of short chain alkane thiol with long chain alkane thiol. Furthermore it was found that OEG addition caused less PEG desorption when compared to alkane thiol. The non-fouling potential of surfaces was tested through serum adsorption and bacterial adhesion studies. We demonstrate that the mixed monolayer with PEG and OEG is better than PEG at resisting protein adsorption and bacterial adhesion, and conclude that backfilling PEG with OEG resulting in the underbrush formation enhances the non-fouling potential of PEG.

KEYWORDS

Non-fouling, PEG, Oligo (ethylene glycol), mixed monolayers, XPS, QCM-D

I. INTRODUCTION

Non-fouling coatings are immensely important to a variety of biomedical applications such as implants, drug delivery, tissue engineering and biosensing. They
are also important for water purification, food processing and marine industries. One goal of a non-fouling coating is to reduce biofilm formation on surfaces while maintaining functionality. Biofilm formation is found to be facilitated by a conditioning layer (adsorbed layer of biomolecules from the surrounding environment), which is followed by reversible physiochemical attachment of microbes. Among the various strategies used in making non-fouling coatings, preventing initial bioadhesion is a popular approach that generally involves the use of a hydrophilic polymeric coating. The concept is to present an interfacial steric barrier between the substrate and the environment in order to prevent conditioning layer dependent biofouling. Polyethylene glycol (PEG) is widely used to functionalize surfaces in order to render them non-fouling. The osmotic and elastic properties of PEG along with its neutral charge, non toxic nature make it an effective and safe choice to minimize fouling in a number of biologically relevant applications. The ability for a given PEG functionalized surface to resist fouling is thought to be mainly dependent on the graft density and molecular weight (MW) of PEG used, both of which are key properties that decide the conformation of PEG chains on surfaces. For example in the case of high or low graft density, PEG chains are arranged in either the ‘brush’ or ‘mushroom’ conformation respectively. A pancake conformation is observed for very low graft densities. Self-assembled monolayers (SAMs) of PEG chains are known to resist non specific adsorption best when present in the brush conformation. Studies have shown that covalently immobilized PEG coatings provide superior antifouling properties, probably since they cannot easily be displaced by biomolecules or conditioning layer molecules. PEG functionalization techniques can be broadly classified into ‘grafting to’ and ‘grafting from’ approaches.
The ‘grafting from’ technique generally gives higher graft density since the limiting factor is diffusion of monomer onto the reactive ends of growing chains whereas in the case of ‘grafting to’ the limitation is diffusion of entire polymer chains to the reactive substrate. Several strategies such as cloud point grafting, grafting in homopolymer solutions, grafting from polymeric melts and underbrush formation by backfilling with shorter molecules have been used to increase the graft density for ‘grafting to’ techniques. The backfilling approach, unlike the other three strategies that depend on minimization of excluded volume interactions, is a simple method wherein the interchain spaces present in layers of high MW PEG chains are filled with shorter PEG chains that can diffuse to the surface. In a study of 5 kDa MW PEG by Uchida et al., it was shown that backfilling with shorter PEG (MW 2 kDa) improved the ability of the surface to resist non specific protein adsorption.

However the mechanism of formation of mixed layers is not yet fully understood. The possibility of the formation of domains exists where the mechanism of addition is similar to that of the replacement phenomena observed for short alkane thiol SAM when they are exposed to longer alkane thiol molecules. Here, replacement of short alkane thiol by longer alkane thiols begins at grain boundaries and is followed by gradual replacement of entire domains, resulting in phase separated arrangement. The replacement of shorter alkane thiols with longer alkane thiols in SAMs is thermodynamically favored due to the higher number of intermolecular Van der Waals interactions formed with longer alkane thiol molecule. Uchida et al. studied the backfilling of high MW PEG layers with low MW PEG and assumed that the addition of low MW species results in formation of well-mixed, homogeneous mixed layers.
The aspect of replacement and the extent of replacement are expected to be low since it is thermodynamically unfavorable for a longer PEG molecule to be replaced by a shorter PEG molecule.

Here we have used oligo (ethylene glycol) (OEG) terminated alkane thiols to backfill PEG thiol SAMs on Au surfaces. Since alkane thiols can form SAMs driven by intermolecular Van der Waals interactions and are kinetically more reactive than high MW PEG thiols our aim is to thoroughly explore whether phase separated domains are formed (Figure 1a) and also the extent of PEG desorption. The schematic presentation of thiolated PEG, OEG and alkane molecules are presented in Figure 1c. Although SAMs of OEG have been shown to be very efficient in resisting protein adsorption \(^{18}\) for short durations, it has been identified that applications involving immobilization of bioactive molecules such as enzymes would benefit from longer PEG chains due to higher mobility for biorecognition \(^{19}\). Thus we expect our combination of PEG-COOH\(_{\text{SAM}}\) backfilled with OEG\(_3\) will be an ideal support for immobilizing bioactive molecules.

We will use the following notations for SAM of PEG and for the backfilled surface:

- PEG-COOH\(_{\text{SAM}}\), OEG\(_3\)-SAM, and R\(_{\text{SAM}}\) correspond to SAMs formed with PEG-COOH, OEG\(_3\), and R molecules
- PEG-COOH\(_{\text{SAM}}\)+OEG\(_3\): PEG-COOH\(_{\text{SAM}}\) backfilled with OEG\(_3\)
- PEG-COOH\(_{\text{SAM}}\)+R: PEG-COOH\(_{\text{SAM}}\) backfilled with R

II. MATERIALS AND METHODS

A. **Chemicals**
α-carboxyl-ω-thiol poly(ethylene glycol) (PEG-COOH, 99% purity) was purchased from Laysan Bio Inc. (Alabama, USA), and Hydroxy-terminated tri (ethylene glycol) undecane thiol (OEG₃, 99% purity) was purchased from Assemblon (Washington, USA). Fetal bovine serum (FBS), octane thiol (R) (99% purity), SYBR Green II, phosphate buffered saline (PBS) buffer tablets, 25% ammonium hydroxide (NH₄OH), 30% hydrogen peroxide (H₂O₂) and absolute ethanol were purchased from Sigma Aldrich (Aarhus, Denmark). Ultrapure MilliQ (MQ) water with resistivity of 18.2 MΩ was used for making buffers and aqueous ethanolic solutions.

B. Substrate preparation and functionalization

Gold substrates were prepared by sputtering 50 nm gold layer onto a 3 nm titanium adhesion layer on silicon wafers. Sputtering was done using a RF sputtering system with Ti and Au targets of 10 cm diameter (2.54 W cm⁻²) inside a standard chamber maintained at an Ar pressure of 2 × 10⁻³ mbar. The substrate-target separation distance was 7 cm, while the deposition rates for Ti and Au were 0.4 nm s⁻¹ and 1 nm s⁻¹ respectively. The gold surfaces were cleaned by UV/ozone treatment for 30 minutes, followed by treatment with basic piranha solution (H₂O: NH₄OH: H₂O₂ in ratio 4:1:1) at 70°-80°C for 5 minutes and then rinsed with MQ water. (CAUTION: Piranha solution reacts violently upon contact with organic solutions). Cleaned gold slides were immersed for 3 h at room temperature in 0.2 mM PEG-COOH solution (75% ethanol), followed by rinsing with MQ water to remove any physically adsorbed molecules. Backfilling involved immersion of PEG-COOH_SAM for 3 h at room temperature in 1mM absolute
ethanolic solution of OEG₃ or R, followed by rinsing with ethanol and drying with a jet of nitrogen. The SAMs of OEG₃ and R for control experiments were made by immersing cleaned gold slides in 1mM ethanolic solution of OEG₃ or R for a period of 3 h followed by rinse with ethanol and drying with nitrogen gas.

C. X-ray photoelectron spectroscopy

XPS spectra were recorded using a Kratos Axis Ultra DLD instrument (Kratos Ltd, Telford, UK) equipped with a monochromated aluminum anode (Al kα 1486 eV) operating at 150 W power (15 kV and 10 mA) with pass energies of 80 eV and 160 eV for high resolution and survey spectra respectively. A hybrid lens mode was employed during analysis (electrostatic and magnetic). The XPS spectra were measured at three areas on each sample and the take-off angle for all measurements was 0°. The measured binding energy positions were charge corrected with reference to 285.0 eV, corresponding to the C-C/C-H species. Quantification and curve fitting was conducted using CasaXPS software. A linear background with a Gaussian to Lorentzian ratio of 30 was used to fit all spectra. The S 2p high resolution spectra were deconvoluted into two doublets corresponding to bound and unbound thiol each with full width half maximum of 1.2 eV). The lower binding energy doublet with its 2p₃/₂ at 161.9 eV was assigned to bound thiol while the higher binding energy doublet with its 2p₃/₂ at 163.5 eV corresponded to unbound thiol as described by Castner et al. The representative high resolution spectra are included in supplementary information (Figure S1b). Overlayer thickness and graft density were calculated using the following equations:

\[
\frac{I}{I_o} = \exp\left(\frac{-d}{\lambda \sin \theta}\right) \quad \text{Eqn-1}
\]
\[ \lambda = \left[ \frac{3.117^{(0.\chi_v^v)} + 0.4207N_{\text{rings}}}{N_{\text{non-H}}} + 1.104 \right] (E_{\text{KeV}})^{0.79} \text{ Eqn-2} \]

\[ L = \left( \frac{M}{\rho N_A d} \right)^{\frac{1}{2}} \text{ Eqn-3a} \]

\[ S = \frac{1}{L^2} \text{ Eqn-3b} \]

Where \( I_0 \) – signal intensity from bare infinitely thick substrate, \( I \) – substrate signal after functionalization, \( d \) – overlayer thickness, \( \lambda \) – attenuation length of Au 4f photoelectron in PEG polymer, \( \theta \) – take off angle of analyzer, \( \chi_v \) – atomic connectivity index of polymer repeat unit, \( N_{\text{rings}} \) – number of aromatic rings, \( N_{\text{non-H}} \) – number of atoms in repeat unit excluding hydrogen atoms, \( E_{\text{KeV}} \) – kinetic energy of photoelectron, \( L \) – interchain distance, \( M \) – molecular weight of PEG chain, \( \rho \) – density assumed to be 1 gm/cm\(^3\), \( N_A \) – Avogadro number, \( S \) – graft density defined as number of chains per unit area.

### D. Contact angle measurements

Static contact angles were measured on all surfaces using water, formamide and \( \alpha \)-bromonapthalene. All images of liquid drops on surfaces were recorded using a KRUSS DSA100 (KRUSS GmbH, Hamburg, Germany), followed by drop shape analysis using ImageJ software. Reported contact angles are the average of at least 6 measurements at different surface positions (data are included in supplementary information Table S1a). The values of \( \gamma^d \), \( \gamma^+ \), \( \gamma^- \) for the three liquids used are given in supplementary information (Table S1b). The interfacial Lifshitz van der Waals (\( \gamma^d \)) and polar components (\( \gamma^+ \), \( \gamma^- \)) of surface tension of a surface can be determined by measuring
contact angles with three different liquids followed by solving the corresponding equations whose general form is given by 22,

$$(1 + \cos \theta)\Delta_L = \frac{1}{2} \left[ (\gamma_S^+ \gamma_L^+ - \gamma_S^- \gamma_L^-) + (\gamma_S^+ \gamma_L^- - \gamma_S^- \gamma_L^+) \right] \quad \text{Eqn 4}$$

$\theta$ – Measured contact angle. $\gamma_L$, $\gamma_L^d$, $\gamma_L^+$ and $\gamma_L^-$ represent total surface tension, Lifshitz van der Waals component, electron acceptor component, electron donor component of liquid respectively. $\gamma_S$, $\gamma_S^d$, $\gamma_S^+$, $\gamma_S^-$ and represent total surface tension, Lifshitz van der Waals component, electron acceptor component, electron donor component of sample surface respectively.

**E. Atomic force microscopy**

All of the AFM images were recorded using a commercial Nanoscope VIII MultiMode SPM system (Bruker AXS, Santa Barbara, CA) in MQ water. Topography images were recorded using Quantitative Nano-Mechanical Mapping (QNM) under Peakforce Tapping mode 23. Ultrasharp silicon nitride cantilevers (triangular, Mpp-12120-10, Bruker AXS) were used with a typical resonance frequency of 150 KHz in air, a spring constant of 5 N/m and a normal tip radius of 8 nm. All AFM images were recorded with $512 \times 512$ pixels resolution per image, and they were flattened and analyzed with Scanning Probe Image Processor software (SPIPTM, Image Metrology ApS, version 5.1.3, Lyngby, Denmark).

**F. Quartz crystal microbalance with dissipation**

QCM-D studies were performed using a Q-Sense E4 system (Gothenburg, Sweden). The PEG-COOH functionalization followed by backfilling with OEG$_3$ or R was
performed on QSX 301 crystals (Q-Sense) coated with 100 nm gold which had approximate resonance frequency of 5 MHz. Serum adsorption experiments were performed in triplicate. Functionalized gold coated crystal surfaces were equilibrated at 37 °C in PBS (100 mM pH 7.4) prior to exposure to serum solution. All serum adsorptions studies were carried out using 10% fetal bovine serum (FBS) at 37 °C. Once a stable baseline was attained, the surfaces were exposed to 750 μL of protein solution delivered at a flow rate of 100 μL/minute followed by rinse with PBS buffer. The shift in resonance frequency corresponding to the 7th overtone was used to monitor the adsorption. After measurement QCM crystals exposed to serum were rinsed with MQ water to remove any salts before drying under a jet of nitrogen for XPS analysis.

**G. Bacterial adhesion assay**

A *Staphylococcus aureus* (ATCC 12598) starter culture was inoculated from agar plates and grown in 3 ml of 1% tryptic soy broth (TSB) medium in 50 ml conical bottom tube by incubating overnight in a shaker at 37 °C. An inoculum prepared by adding 1 ml of starter culture to 100 ml 1% TSB medium was incubated in a shaker at 37 °C until cultures were in the late-exponential growth phase (OD600 = 1.0). Cells were then harvested by centrifugation (5 minutes at 3000 x g), washed twice and resuspended in PBS (pH 7.4) and diluted to obtain an OD600 of 0.5. Surfaces were incubated with the bacterial suspensions in 24 well plates for 5 h at 37°C and gently washed three times with sterile PBS to remove non-adherent cells. The adherent cells on the surfaces were stained with ~10 μl 20x SYBR Green II RNA stain (2 μl ml⁻¹ of 10,000x SYBR Green II stock), covered with glass cover slips and sealed with nail polish to avoid evaporation. Slides were stored in the dark at 4 °C until analyzed. Adherent cells were counted by
fluorescence microscopy using a Zeiss Axiovert 200M epifluorescence microscope (Carl Zeiss GmbH, Jena, Germany) equipped with Zeiss filter set 10 and 63x oil immersion objective. Cells were counted in 109 µm² grids on triplicates of sample on a total of fifteen random positions on each of the surface type.

III. RESULTS AND DISCUSSION

A. Extent of addition and coverage in backfilled monolayer

XPS was used to monitor the chemical changes occurring on Au substrates functionalized with PEG-COOH upon exposure to OEG₃ or R molecules. The respective relative elemental and chemical species calculated from survey and high resolution XPS scans are summarized in Table 1. The substrate photoelectrons are attenuated by the overlayer and hence the substrate signal has an inverse relationship with overlayer coverage as shown in Eq-1. The Au 4f signal was used to obtain quantitative data on the addition of molecules to the PEG-COOHₐₐₘ. The Au 4f signal of PEG-COOHₐₐₘ+OEG₃ is 3.6 % lower than that of PEG-COOHₐₐₘ, indicating that OEG₃ molecules have added to the PEG-COOHₐₐₘ. From the chemical structure of OEG₃ and R molecules it can be observed that they have lower O/C and higher alkane (285.0 eV)/PEG (286.7 eV) ratios when compared to PEG-COOH and hence any addition during backfilling results in a corresponding change in the detected O/C and alkane/PEG ratios. The alkane/PEG ratio was calculated from the peak areas of components corresponding to binding energies 285.0 eV (alkane) and 286.7 eV (PEG) obtained from high resolution C 1s spectra shown in supplementary information Figure S1a. Addition of OEG₃ is further supported by a decrease in O/C ratio from 0.49 to 0.42 and increase in alkane/PEG ratio from 0.1 to 0.3 as shown in Table 1. The PEG-COOHₐₐₘ+R system exhibits a decrease in O/C ratio
from 0.49 to 0.40 and increase in alkane/PEG ratio from 0.1 to 0.4 similar to PEG-COOH$_{SAM}$+OEG$_3$ except for the Au signal which increased by 2.6 %. The increase in substrate signal indicates a decrease in overlayer thickness that could be caused by desorption of some PEG-COOH molecules during backfilling by R molecules.

Based on O/C ratios obtained from XPS of PEG-COOH$_{SAM}$, OEG$_3$-SAM, and R$_{SAM}$, a change in O/C ratio as a function of relative coverage of PEG-COOH and OEG$_3$ or R was plotted assuming a linear relationship (Figure 2). By correlating the experimentally obtained values of O/C ratios for PEG-COOH$_{SAM}$+OEG$_3$ and PEG-COOH$_{SAM}$+R, we estimate the coverage of OEG$_3$ or R on the backfilled PEG-COOH$_{SAM}$ surface to be 29% and 18% respectively. It must be noted that this estimation assumes that the intermolecular spaces in PEG-COOH$_{SAM}$ ideally are free for any contamination free, which in real laboratory conditions is not the case and thus we overestimate the number of added molecules. Here, however, we mainly focus on the relative differences rather than the absolute values. Despite the greater number of OEG$_3$ molecules being added to the PEG-COOH$_{SAM}$, the decrease in carboxyl peak area (Table 1) in C1s spectra of PEG-COOH$_{SAM}$+OEG$_3$ and PEG-COOH$_{SAM}$+R are comparable. This suggests that desorption of PEG-COOH is lower when OEG$_3$ is added in comparison to when R is added. It is known that relative atomic percentage of S from SAMs of PEG thiol depends not only on the stoichiometric ratio but also on the extent of dehydration and collapse of brushes during grafting of chains as described by Larry D. Unsworth et al. The amount of S in PEG-COOH$_{SAM}$ was 2.1 %, which is higher than the corresponding stoichiometric ratio of <1%, very similar to what was reported by Unsworth et al. with hydroxyl terminated 2 kDa PEG thiol molecules under cloud point conditions. Though R and OEG$_3$ molecules
have S/C ratios of 0.11 and 0.045 respectively, which is higher than the ratio for PEG-COOH_{SAM} (0.008), the backfilling of PEG-COOH_{SAM} with either R or OEG_{3} did not significantly change the relative atomic percentage of S (Table 1). Hence the S content was not helpful in providing additional information about the backfilling process. The percentages of bound thiol on functionalized surfaces were determined as described above in the methods section 2.3. The amount of bound sulfur was found to increase by a small extent (increase of 3.5%) upon backfilling PEG-COOH_{SAM} with R, and for OEG_{3} it increased by 1.6%. This small increase in bound sulfur could be due to Au-thiol bond formation of backfilled molecules, also indicating that a significant proportion of added molecules have chemisorbed to the gold surface and not physically adsorbed. The deconvoluted high resolution S2p spectra of various samples are included in the supplementary information (Figure S1b).

B. Arrangement of backfilled molecules

As discussed earlier, backfilling process could result in mixed monolayer with two possible arrangements namely underbrush and island. We used contact angle based surface energy measurements to find out the arrangement of backfilled molecules. Contact angle measurements have very high surface sensitivity (<1nm) \textsuperscript{24} and allow the determination of the surface energy of materials \textsuperscript{22}. The components of surface tension can be calculated by measurement of contact angles of three different liquids using Eq- 4 \textsuperscript{22}. The contact angles measured for various surfaces using three liquids are given in the supplementary information (Table S1b). The surface tension components; Lifshitz van der Waals component (\(\gammaS^d\)), electron acceptor component (\(\gammaS^+\)), electron donor component (\(\gammaS^-\)) for various surfaces are shown in Table 2. The hydrophobicity of a surface is
determined by its $\gamma_{S}^{d}$ value, and the lower the value, the more hydrophobic a surface is.

Since alkane thiol molecules are hydrophobic a SAM of alkane thiol would have lower $\gamma_{S}^{d}$ value than PEG or OEG based SAMs and this is apparent for the values determined for $R_{SAM}$ and OEG$_{3-SAM}$ (Table 2). If alkane thiol molecules form islands instead of being homogeneously mixed during the backfilling of the PEG-COOH$_{SAM}$, then the $\gamma_{S}^{d}$ value of PEG-COOH$_{SAM+R}$ would significantly decrease. The $\gamma_{S}^{d}$ value $44.0$ mJ/m$^{2}$ for PEG-COOH$_{SAM+R}$ is virtually the same as that of PEG-COOH$_{SAM}$ that has a $\gamma_{S}^{d}$ value $44.0$ mJ/m$^{2}$ indicating that hydrophobic islands are not present. The $\gamma_{S}^{-}$ value of a surface gives a quantitative idea about the presence of electron donor groups, with the greater the value of $\gamma_{S}^{-}$ the higher the number of surface electron donor groups. PEG and OEG have oxygen atoms and thus have high $\gamma_{S}^{-}$ values when compared to the alkane thiol (Table 2). Backfilling PEG-COOH$_{SAM}$ with R results in a $7$ mJ/m$^{2}$ decrease in $\gamma_{S}^{-}$ indicating that a significant number of PEG-COOH molecules have desorbed from the surface. The values for $\gamma_{S}^{d}$ and $\gamma_{S}^{-}$ for PEG-COOH$_{SAM}$ after addition of OEG$_{3}$ do not change significantly since both molecules have very similar values and could not easily be followed by contact angle measurements.

**C. AFM results**

QNM-AFM has a very high lateral resolution and has been used to characterize the surface morphology of the different SAM layers with the aim to check the presence of phase separated domains. With the use of the peak forces less than $1$ nN, QNM AFM is able to map mechanical properties of surfaces quantitatively while simultaneously capturing topographic images with the same resolutions as traditional tapping mode AFM
All QNM-AFM measurements were done liquid condition. The topography and modulus images of Au surface and various SAM are shown in Figure 3 (Figure 3), and the corresponding surface roughness is summarized in Table 3. The topography of the bare Au surface (Figure 3a) shows Au grains without any additional features on top of grains. The recorded nanomechanical map of Au surface (Figure 3a’) demonstrates the homogeneous Young’s modulus distribution as the whole image has nearly the same range of stiffness showing the same color contrast. Comparing the topography images of modified surfaces with the bare Au, one can clearly see the additional particle-like features on all the modified surfaces (Figure 3b-f). In addition, the modulus images of the modified surfaces show that many of the grain boundaries are slightly invisible due to the SAMs. Furthermore, the surface roughness has been changed dramatically after modification. Hence these evidences prove the successful formation of the SAMs. Interestingly, the modulus of the modified surfaces also represents the surface modulus variations, because of the slightly different color contrast (Figure 3b’-3f’). The small variations in material properties observed in elastic modulus measurements are particularly useful in the analysis of heterogeneous surface. However most importantly, phase separations were not observed in all of images. This indicates that the backfilled surfaces are homogeneous. Hence we conclude that the SH-R-OEG₃ or SH-R molecules form underbrush upon addition to SH-PEG-COOHₐₐₜ.m.

D. Non-fouling properties of PEG-COOH surfaces with and without underbrush

Resistance towards non specific adsorption can be tested using simple model systems such as single protein solutions. Examples have included collagen and bovine
serum albumin\textsuperscript{27}. More rigorous tests are conducted with serum\textsuperscript{27} or microbial cell suspensions\textsuperscript{28}. The non-fouling properties of the PEG-COOH\textsubscript{SAM} and mixed surfaces were evaluated by serum adsorption and bacterial adhesion studies. Addition of smaller OEG chains in theory increases the graft density protein resistance molecules within the layer of diffuse PEG-COOH\textsubscript{SAM} molecules and hence is expected to improve its resistance towards non specific adsorption.

QCM and XPS have been employed to quantitatively compare the serum adsorption resistance properties of the SAMs. The frequency shift after serum exposure in QCM is directly proportional to the amount of protein adsorbed\textsuperscript{27}, and the relative atomic percentage of nitrogen from XPS also enables a quantitative estimation of the amount of adsorbed protein\textsuperscript{26}. In Figure 4a we show the data points corresponding to various samples studied, where the X-axis shows the \% N obtained from XPS and the Y-axis shows the frequency shift from the QCM data. Upon serum exposure the QCM frequency decreases by 7Hz for the PEG-COOH\textsubscript{SAM}+OEG\textsubscript{3} surface, compared to the PEG-COOH\textsubscript{SAM} and PEG-COOH\textsubscript{SAM}+R surfaces, which both exhibit a frequency shift of about 15Hz. The lower frequency shift for PEG-COOH\textsubscript{SAM}+OEG\textsubscript{3} proves that OEG\textsubscript{3} addition improves the non-fouling abilities of PEG-COOH\textsubscript{SAM} surfaces. We also speculate that protein adsorption would be lower if the PEG thiol molecules used did not have a carboxyl end-group, increasing electrostatic interactions with oppositely charged serum proteins and facilitating higher adsorption. Representative QCM kinetic plots are included in the supplementary information (Figure S2). Figure 4a also shows the nitrogen content on the X-axis, where the PEG-COOH\textsubscript{SAM}+OEG\textsubscript{3} surface has 2\% N compared to PEG-COOH\textsubscript{SAM} and PEG-COOH\textsubscript{SAM}+R surfaces both showing approximately 5\% N,
which follows the same trend as seen with QCM. The OEG$_{3-SAM}$ had the highest ability to resist serum adsorption which agrees with previous studies with OEG based SAMs.$^{18}$

The surfaces were tested for bacterial adhesion using a *Staphylococcus aureus* strain and the results are shown in Figure 4b. Clearly, the PEG-COOH$_{SAM+OEG_3}$ ($4.2 \times 10^7$ cells/cm$^2$) surface is better at resisting bacterial adhesion when compared to PEG-COOH$_{SAM}$ ($7.2 \times 10^7$ cells/cm$^2$) and PEG-COOH$_{SAM+R}$ ($9.2 \times 10^7$ cells/cm$^2$). Hence it can be concluded that adding OEG$_3$ molecules improve the non-fouling properties of the PEG-COOH$_{SAM}$. Though the OEG$_{3-SAM}$ has the least bacterial adhesion and serum adsorption, we propose that PEG-COOH$_{SAM+OEG_3}$ system would be a better system for applications involving immobilization of bioactive molecules such as enzymes and further advantages of PEG-COOH$_{SAM+OEG_3}$ will be discussed in the next section.

**E. Discussion**

We have studied mixed monolayers of OEG$_3$ on PEG-COOH$_{SAM}$ using XPS, contact angle and AFM. The contact angle results support the notion that phase separated surface arrangement of the two molecules does not occur proving that homogeneous mixed layers are formed. There have been some reports on grafting 2 kDa PEG thiol chains. For example Unsworth et al.$^8$ reported a maximum graft density of 0.58 chains/nm$^2$ using methoxy capped 2 kDa PEG thiol chains under cloud point conditions, and Tokumitsu et al.$^{29}$ reported a maximum graft density of 3.6 chains/nm$^2$ using 2 kDa PEG conjugated to undecane thiols. From our XPS data we have calculated a graft density of PEG-COOH$_{SAM}$ to be 1.0 chain/nm$^2$ using Eq 1-3. Thus, the PEG-COOH chains in PEG-COOH$_{SAM}$ have a density that is not close-packed and there exists
sufficient space for the smaller OEG molecules to fill the gaps, although in a good solvent the PEG is likely to extend and form highly mobile brushes. We used Carboxyl capped PEG molecules since we intend to use them for immobilization of antibacterial enzymes in future studies. These molecules are expected to contribute to the surface negative charge. We suspect that the non-fouling properties would be better with hydroxyl or methoxy capped PEG molecules, which are neutrally charged and thus minimize electrostatic interactions. Our mixed PEG-COOH$_{\text{SAM}^+}$OEG$_3$ system is anticipated to be an ideal platform for immobilization of bioactive molecules, since the OEG maximizes non-fouling potential while the longer PEG chains would confer higher mobility and thus improved bioactivity capabilities to the immobilized molecule. There have been reports on bacterial adhesion studies on OEG and PEG surfaces. Our adhesion studies with Staphylococcus aureus involved different incubation time compared to that used by Nejadnik et al., also Ostuni et al. used a counting technique involving removal of attached cells by sonication followed by counting the colony forming units on culture plates that introduces possibility of 10 fold error in estimation as mentioned by authors. Hence the absolute values of our bacterial adhesion studies could not be compared with these earlier reported studies. We plan to immobilize enzymes on backfilled PEG-COOH$_{\text{SAM}^+}$OEG$_3$ surfaces and further understand the role of backfilled OEG molecules.

**IV. SUMMARY AND CONCLUSIONS**

We have investigated backfilling of a PEG-COOH$_{\text{SAM}}$ with alkane and oligo ethylene glycol terminated thiol molecules. From XPS and contact angle results we have
shown that R molecules form mixed layers and the addition process is accompanied by desorption of PEG-COOH molecules. OEG₃ molecules, upon addition, also formed mixed layers but less desorption of the PEG occurs, when compared to addition of R molecules. The non-fouling properties of various surfaces were compared, and it was shown that the PEG-COOHₛₐₐₘ₊OEG₃ surface found to be better than the PEG-COOHₛₐₐₘ surface with respect to resisting serum adsorption and bacterial adhesion. Thus, the strategy involving backfilling using OEG terminating alkane thiols is indeed capable of improving the non-fouling properties of monolayers of PEG chains of significant graft densities.

ACKNOWLEDGMENTS

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Table 1. Summary of XPS results. Au % - relative atomic percentage of Au. O/C - ratio of relative atomic percentage oxygen and carbon. Alkane (285.0 eV)/PEG (286.7 eV) - ratio of alkane and PEG components in C 1s high resolution spectra. S % - relative atomic percentage of S. % bound S - percentage of bound sulfur from components in S 2p high resolution spectra.

<table>
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<tr>
<th>Sample</th>
<th>PEG-COOH&lt;sub&gt;SAM&lt;/sub&gt;</th>
<th>OEG&lt;sub&gt;3&lt;/sub&gt;-SAM</th>
<th>R&lt;sub&gt;SAM&lt;/sub&gt;</th>
<th>PEG-COOH&lt;sub&gt;SAM&lt;/sub&gt;+R</th>
<th>PEG-COOH&lt;sub&gt;SAM&lt;/sub&gt;+OEG&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au %</td>
<td>39.0±0.4</td>
<td>32.6±0.4</td>
<td>55.3±2.4</td>
<td>41.6±0.5</td>
<td>36.2±0.5</td>
</tr>
<tr>
<td>O/C</td>
<td>0.49±0.03</td>
<td>0.25±0.01</td>
<td>0.0±0.0</td>
<td>0.40±0.01</td>
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<td>Alkane/PEG</td>
<td>0.1±0.0</td>
<td>0.8±0.0</td>
<td>21.0±0.0</td>
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<tr>
<td>S %</td>
<td>2.1±0.2</td>
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<tr>
<td>% bound S</td>
<td>78.3±0.7</td>
<td>80.7±1.0</td>
<td>88.1±1.0</td>
<td>81.8±1.0</td>
<td>79.9±0.7</td>
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Table 2. Surface tension components: the Lifshitz van der Waals component ($\gamma_S^d$), electron acceptor component ($\gamma_S^+$), and electron donor component ($\gamma_S^-$) determined from the surfaces of OEG<sub>3</sub>-SAM, R<sub>SAM</sub>, PEG-COOH<sub>SAM</sub>, PEG-COOH<sub>SAM</sub>+R and PEG-COOH<sub>SAM</sub>+OEG<sub>3</sub>.
<table>
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<tr>
<th>Sample</th>
<th>$\gamma_s^d$ mJ/m$^2$</th>
<th>$\gamma_s^+ mJ/m^2$</th>
<th>$\gamma_s^- mJ/m^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OEG$_3$-SAM</td>
<td>43.45±0.06</td>
<td>0.85±0.05</td>
<td>45.66±1.49</td>
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<tr>
<td>RSAM</td>
<td>30.67 ± 1.27</td>
<td>0.01±0.01</td>
<td>0.69±0.26</td>
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<td>PEG-COOH$_3$-SAM</td>
<td>44.24±0.04</td>
<td>0.92±0.05</td>
<td>43.97±1.56</td>
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<td>PEG-COOH$_3$-SAM+R</td>
<td>44.05±0.08</td>
<td>0.96±0.03</td>
<td>36.58±0.80</td>
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<tr>
<td>PEG-COOH$_3$-SAM+OEG$_3$</td>
<td>44.07±0.1</td>
<td>0.90±0.05</td>
<td>42.90±1.59</td>
</tr>
</tbody>
</table>

**Table 3:** Normalized Surface roughness (to Au) values for OEG$_3$-SAM, RSAM, PEG-COOH$_3$-SAM, PEG-COOH$_3$-SAM+R and PEG-COOH$_3$-SAM+OEG$_3$. $S_{SAM}/S_{Au}$ stands for ratio of the means of the two surface roughness; $S_{Au}= 0.48±0.02\text{nm}$

<table>
<thead>
<tr>
<th>Sample</th>
<th>Roughness (Sq) (nm)</th>
<th>$S_{SAM}/S_{Au}$</th>
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</thead>
<tbody>
<tr>
<td>PEG-COOH$_3$-SAM</td>
<td>0.69±0.04</td>
<td>1.44</td>
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<tr>
<td>OEG$_3$-SAM</td>
<td>0.63±0.04</td>
<td>1.31</td>
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<tr>
<td>RSAM</td>
<td>0.21±0.02</td>
<td>0.44</td>
</tr>
<tr>
<td>PEG-COOH$_3$-SAM+OEG$_3$</td>
<td>0.81±0.05</td>
<td>1.69</td>
</tr>
<tr>
<td>PEG-COOH$_3$-SAM+R</td>
<td>0.66±0.06</td>
<td>1.38</td>
</tr>
</tbody>
</table>

**Figure Captions**

Figure 1. (Colour online only) The schematic diagrams of back filled PEG SAM in island type (a) and underbrush type arrangements (b). The chemical formula and assigned abbreviations for various molecules used in self assembly and backfilling experiments (c).

Figure 2. (Colour online only) Relative coverage of PEG-COOH and R or OEG$_3$ on backfilled surfaces as determined by correlating their XPS O/C ratios (on X axis) to a plot obtained by extrapolating values of O/C ratio for 0% and 100% relative coverage (on Y axis) assuming a linear relationship. The data points ▲ and + shown in the plot.
represent the experimentally obtained O/C ratios for PEG-COOH$_{\text{SAM}}$+OEG$_3$ and PEG-COOH$_{\text{SAM}}$+R respectively.

Figure 3. (Colour) Topography and Young’s modulus images recorded by QNM-AFM measurements in MQ water. (a, a’) Au substrate, (b, b’) PEG-COOH$_{\text{SAM}}$, (c, c’) OEG$_3$$_{\text{SAM}}$, (d, d’) RSAM, (e, e’) PEG-COOH$_{\text{SAM}}$+OEG$_3$, and (f, f’) PEG-COOH$_{\text{SAM}}$+R. The scan area of each image is 1µm×1µm.

Figure 4. (Colour) Non-fouling properties of the surfaces. (a) Shows XPS (X-axis – N %) and QCM (Y-axis – frequency shift) based quantification after exposure to 10% FBS adsorption, to PEG-COOH$_{\text{SAM}}$, PEG-COOH$_{\text{SAM}}$+R and PEG-COOH$_{\text{SAM}}$+OEG$_3$, OEG$_3$$_{\text{SAM}}$ and RSAM surfaces. (b) Shows results of bacterial adhesion studies using Staphylococcus aureus performed on PEG-COOH$_{\text{SAM}}$, PEG-COOH$_{\text{SAM}}$+R and PEG-COOH$_{\text{SAM}}$+OEG$_3$, OEG$_3$$_{\text{SAM}}$, RSAM. The X-axis shows the number of adherent cells on the Log scale.

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