Hydrogel coatings for biomedical and biofouling applications

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During the course of the research underlying this thesis, Tobias Ekblad was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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To Yi-Chi.
Many applications share a substantial and yet unmet need for prediction and control of interactions between surfaces and proteins or living cells. Examples are blood-contacting biomaterials, biosensors, and non-toxic anti-biofouling coatings for ship hulls. The main focus of this thesis work has been the synthesis, characterization and properties of a group of coatings, designed for such applications. Many types of substrates, particularly plastics, were coated directly with ultrathin, hydrophilic polymer coatings, using a newly developed polymerization method initiated by short-wavelength ultraviolet light.

The thesis contains eight papers and an introduction aimed to provide a context for the research work. The common theme, discussed and analyzed throughout the work, has been the minimization of non-specific binding of proteins to surfaces, thereby limiting the risk of uncontrolled attachment of cells and higher organisms. This has mainly been accomplished through the incorporation of monomer units bearing poly(ethylene glycol) (PEG) side chains in the coatings. Such PEG-containing “protein resistant” coatings have been used in this work as matrices for biosensor applications, as blood-contacting inert surfaces and as anti-biofouling coatings for marine applications, with excellent results. The properties of the coatings, and their interactions with proteins and cells, have been thoroughly characterized using an array of techniques such as infrared spectroscopy, ellipsometry, atomic force microscopy, surface plasmon resonance and neutron reflectometry. In addition, other routes to fabricate coatings with high protein resistance have also been utilized. For instance, the versatility of the fabrication method has enabled the design of gradients with varying electrostatic charge, affecting the protein adsorption and leading to protein resistance in areas where the charges are balanced.

This thesis also describes a novel application of imaging surface plasmon resonance for the investigation of the surface exploration behavior of marine biofouling organisms, in particular barnacle larvae. This technique allows for real-time assessment of the rate of surface exploration and the deposition of protein-based adhesives onto surfaces, a process which was previously very difficult to investigate experimentally. In this thesis, the method was applied to several model surface chemistries, including the hydrogels described above. The new method promises to provide insights into the interactions between biofouling organisms and a surface during the critical stages prior to permanent settlement, hopefully facilitating the development of anti-biofouling coatings for marine applications.


Det här arbetet har bedrivits inom ramen för ett europeiskt forskningsprojekt som har som mål att hitta metoder för att minska marin påväxt med moderna metoder, utan att använda gifter. I princip handlar det om att förstå och motverka de processer som leder till att t.ex. havstulpaner sätter sig fast på ytor. En metod som vi arbetat med i det här arbetet har varit att designa ytor som inte tillåter att havstulpanens klister fastnar, då den söker efter en lämplig plats att slå sig ned för resten av livet. Tanken är att larven då helt enkelt ska simma bort från skrovet och hitta någon mer bekväm, och för båtägaren mer fördelaktig, plats att sätta sig. För att kunna undersöka om den här idén fungerar har vi även utvecklat en ny teknik för att studera hur havstulpanlarver undersöker ytor innan de sätter sig fast. Vi har använt oss av tekniken ytplasmonresonans för att undersöka hur larven med sina "fötter" (de främsta delarna av ett antennpar, som larven använder för att undersöka sin omgivning) promenerar omkring över ytan och lämnar mikrometerstora "fotavtryck". Med hjälp av tekniken, som är extremt ytkenstig, kan man i direkt och i realtid se både larvernas "fotsulor" och "fotavtryck" underifrån. Huruvida det blir några avtryck eller inte verkar bero på ytkemin, vilket är lovande eftersom det tyder på att man kanske kan lura havstulpanen genom att tillverka en ogiftig bottenfärg som har en yta som larven inte tycker om, eller som den inte klarar av att sätta sig fast på. Även om steget är långt till en effektiv färg som fungerar efter de här principerna, är det viktigt att ha verktyg för att förstå hur havstulpanlarverna reagerar på ytan egenskaper. Vår nya metod är ett sådant verktyg, som förhoppningsvis kan leda till nya upptäckter inom det här området.
Ytans effekt har också studerats i andra biologiska sammanhang, närmare bestämt för biomaterial i blodkontakt. Många material används rutinmässigt i kontakt med blod, men egentligen finns det fortfarande inget riktigt bra sätt att förhindra att blodet reagerar med ytan på olika sätt, vilket kan leda till negativa konsekvenser, både för materialet och för blodet. Vi har studerat hur man kan göra ytor mindre benägna att aktivera blodkoagulation och hur man kan minska risken att blodplättar sätter sig fast. Problemställningen har alltså vissa likheter med påväxtprojektet, och kanske gäller det också lösningen – i båda fallen har det visat sig att material som kraftigt minska bindningen av proteiner till ytan är användbara.
ACKNOWLEDGEMENTS

Looking at the list of papers, and remembering all the work behind them, and all the work that never (yet at least) ended up in a paper, it strikes me how much of a team effort this has been. Consequently, I have a lot of people to thank.

Primarily, I would like to thank Bo Liedberg, who has been my main supervisor and who has been extremely tolerant and generous, allowing me to keep chasing all those far-fetched hydrogel-related goals, which he probably understood could never quite be reached. In a few cases I managed to find the answers on my own, in other cases I received the help I needed to return to the right track. I am grateful for that experience.

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Then, I would like to thank all those who I have collaborated and cooperated with in Linköping: Lars Faxälv, with whom I have one of the longest running PhD collaborations imaginable, extending over several years and self-imposed deadlines (I guess we had too much fun to quit). Olof Andersson, who I have, in all fairness, relied on a little bit too many times, and who has always had an answer to my questions. András Larsson, for a fruitful collaboration which combined the best (I hope) of two personalities. Feng-I Tai, for all those AFM measurements and for a lot of good discussions. Gunnar Bergström, for toiling very hard with the hydrogels early on, before moving on to more varied work. Magnus Falk and Nils Odelstam for contributing to the development of the grafting method and Chun-Xia Du for making those masks which were needed. Tomas Lindahl for patiently putting up with me and Lars. Ye Zhou, Patrik Nygren, Mattias Östblom and Timmy Fyrner for working hard with AMBIO-related questions. Hanna Lassus, Andrea Gambino, Anna Bergström, Alberto Mangone, Nanny Wallmark and Olof Sterner, all of whom past Master’s students who I supervised to a major or minor extent, and who gave me much inspiration and many new ideas. Also, many of the students I have supervised in project courses have given me thought-provoking questions, for which I am thankful.

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This thesis is based on the following eight papers, which are referred to in the text by their Roman numerals.

**Paper I**
Larsson A, Ekblad T, Andersson O and Liedberg B.  
*Biomacromolecules, 2007*, (8), 1, 287-295.

**Paper II**
Poly(ethylene glycol)-Containing Hydrogel Surfaces for Antifouling Applications in Marine and Freshwater Environments.
*Biomacromolecules, 2008*, (9), 10, 2775-2783.

**Paper III**
Lateral Control of Protein Adsorption on Charged Polymer Gradients.
Ekblad T, Andersson O, Tai F-I, Ederth T and Liedberg B.  

**Paper IV**
Blood Compatibility of Photografted Hydrogel Coatings.
Faxälv L, Ekblad T, Liedberg B and Lindahl TL.  

**Paper V**
Patterned Hydrogels for Controlled Platelet Adhesion from Whole Blood and Plasma.
Ekblad T, Faxälv L, Andersson O, Wallmark N, Larsson A, Lindahl TL and Liedberg B.  
*Submitted*. 


Paper VI
Swelling of Grafted Poly(ethylene glycol)-Containing Hydrogels – a Neutron Reflectivity Study.
Ederth T and Ekblad T.
In manuscript.

Paper VII
Novel Application of Imaging Surface Plasmon Resonance for in situ Studies of the Surface Exploration of Marine Organisms.
Andersson O, Ekblad T, Aldred N, Clare AS and Liedberg B.

Paper VIII
In situ Quantification of Surface Exploration and Footprint Deposition by Barnacle Cyprids (Semibalanus balanoides) using Imaging Surface Plasmon Resonance.
Aldred N, Ekblad T, Andersson O, Liedberg B and Clare AS.
In manuscript.

The following publications were not included in this thesis.

Gradient Hydrogel Matrix for Microarray and Biosensor Applications: An Imaging SPR Study.
Andersson O, Larsson A, Ekblad T and Liedberg B.

Interactions of Zoospores of Ulva linza with Arginine-rich Oligopeptide Monolayers.

Hydrogel Gradients by Self-initiated Photografting and Photopolymerization: Preparation, Characterization and Protein Interactions.
Ekblad T, Larsson A and Liedberg B.
Submitted as a contribution to the book “Soft Matter Gradient Surfaces: Methods and Applications“.
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1 GENERAL INTRODUCTION

The selection of a suitable material for a specific application is often based on a few criteria, commonly including availability, cost, and whether the material can fulfill the most essential physical requirements. Further demands often need to be dealt with through makeshift solutions and compromises. Let us illustrate this with the example of ship hulls. Ship hulls were, for a very long time in human history, almost exclusively made of wood. The availability was generally good (although problematic for larger vessels), the cost was high (but had to be dealt with since there were no realistic alternatives), and the mechanical strength was sufficient to allow the construction of relatively large ships, which were capable of sailing round the world. There were problems too, of course: wooden ships were fragile, especially in combat situations, and ship worm, fouling of the bottom and dry rot were regarded as constant evils.[1] Some of these problems were partially solved by attaching metal plates onto the outside of the hull. Thus, a basic material (wood) was used together with a “coating” (iron or copper plates) to achieve a better effect than with the basic material alone. When the steam engine and propeller were introduced, it became evident that greater mechanical strengths were necessary to accommodate these previously unseen powers of propulsion, and ships built completely of iron were therefore introduced. Once the primary demands of sufficient mechanical integrity and safety and been accommodated, concerns of secondary importance had to be resolved. The iron hulls corroded rapidly in the marine environment, and were not compatible with the methods developed to prevent fouling of wooden hulls.[1] New adaptations and compromises were necessary. Again, the solutions were found, or at least sought, in the use of coatings, which were adopted to protect and supplement the properties of the bulk material. When combined, the coating and the underlying material could perform far better than any of the individual components alone. The coating handled the interface to the surrounding world, while the underlying material was allowed to carry on doing whatever it was designed to do. This versatile approach remains successful to this day, for ships as well as in many other applications. The main challenge lies in designing the coating, so that it both meets the required interfacial demands and can be securely applied to the underlying material, for as long as it is needed.

This work describes the development, characterization and performance of ultrathin hydrogel coatings, possible to apply to a range of substrates. Before we go any further, it might be good to define exactly what is meant by “hydrogel coating” in this work, and to provide some other definitions which help understanding the contents of this thesis.

A polymer is a large macromolecule made up of smaller units, so-called monomers. Most commonly used synthetic polymers, such as polyethylene or polystyrene, are non-polar and hydrophobic.[2] However, if the monomer units contain polar or charged groups the polymer
may be water-soluble. Polymers of this type possess several attractive properties for biological applications, mainly due to the strongly associated water molecules which “cling” to the polymer chains. These properties might include high biocompatibility, resistance to protein adsorption, softness, and a high diffusion rate of dissolved molecules. In some biomedical applications, notably contact lenses, these properties have enabled the successful use of materials composed entirely of cross-linked hydrophilic polymers. Materials of this type are commonly referred to as hydrogels.\[^3\] In this work, the definition of the term hydrogel is widened somewhat, to include hydrogel-like thin films prepared as coatings on supporting substrates. The motivation for using the term “hydrogel” is that the films share many properties with conventional hydrogels, including a certain degree of cross-linking. Another useful term, which is commonly used to describe similar coatings, is polymer brush. It is used to describe dense but thin polymer films, which are not cross-linked but are anchored at one end to a substrate.\[^4\] The most suitable word for the hydrogels prepared in this work might be “bush”, rather than “brush”.

Hydrogels are generally mechanically too weak (low modulus, low tensile strength) for many applications, which has restricted the use of these materials. In addition, certain applications may demand specific optical or electrical properties which cannot be provided by hydrogels. By applying a thin hydrogel-like coating onto the surface of a material with suitable bulk properties, it may be possible to exploit e.g. the biocompatibility of hydrogels, without sacrificing other necessary material properties. That is, you let the hydrogel handle the interfacial interactions with the surrounding (biological) world. The highly generic term coating will, in this work, primarily describe very thin coatings, on the nanometer thickness scale. These coatings are often made by grafting to or from a substrate. The term “grafting” is taken from the world of horticulture, where it describes the process of fusing the shoots of one plant with the rootstock of another plant.\[^5\] In polymer science, the term describes the fusing of different polymer chains, or more generally, the fusing of polymer chains to a solid substrate.[^2]

In this work, the most central property handled by the hydrogel coatings is resistance to non-specific adsorption of biomacromolecules, primarily proteins. Surfaces which display such properties may be referred to as nonfouling\[^6\] or protein resistant.\[^7\] This is an unusual and potentially also very useful property, which has been studied intensely over the last decades. To put the work into context, much of the introductory part of the thesis is constructed as a relatively extensive review of why protein resistance is important and how protein resistant coatings can be fabricated. The actual research carried out is described in detail in the papers at the end of the thesis, and is also commented and briefly described in Chapter 5.
The following chapter will outline two applications in which coatings demonstrating protein resistance and low adhesion of cells and organisms are, or are expected to become, very important. In addition, the currently available strategies for achieving protein resistance will be described. The challenges of developing suitable testing methods are also discussed at the end of the chapter.

2.1 Marine and freshwater biofouling

The sea is not only full of water, but also of solid surfaces. Many organisms – bacteria, algae, worms, mollusks and barnacles – have found a biological niche in attaching themselves to these surfaces at some point in their life cycle. There, they can extract nutrients from the surrounding water in a convenient and energy-efficient manner. This way of life has led to the evolutionary development of some rather unique features amongst the sessile organisms, particularly the ability to synthesize different glues which enable the organisms to adhere strongly to a range of surfaces. In addition, sessile organisms often have a hard, calcareous shell for protection against predators, crashing waves, and dehydration. If an object is submerged in the sea, its surface will almost instantaneously be colonized by opportunistic sessile organisms. This process starts with the adsorption of organic compounds, biomacromolecules and ions (so-called conditioning), followed by the rapid accumulation of bacteria and other single-cell organisms, in particular diatoms, which form colonies which eventually cover the surface completely. This biofilm contains not only the organisms themselves but also a large amount of excreted biomacromolecules, giving it a slime-like structure. The ecological succession proceeds with the settlement of algal spores and larvae of sessile animals, such as barnacles and mussels. Eventually, as the animals and alga grow larger, the whole surface may be covered with a relatively thick layer of living organisms (see Figure 2.1 and 2.2 for examples). The ecology of the system will depend greatly on the surrounding environment, for instance the water temperature, light intensity, salinity and the local availability of larvae and spores. Similar processes take place in freshwater, albeit normally without the dramatic growth often encountered in marine environments.

When this process takes place on a submerged foreign object, such as a ship hull or an optical window for an underwater instrument, it is referred to as marine biological fouling, biofouling, or simply “fouling”. It is a major problem in most marine-related industries as
well as in many freshwater applications. The most well-known and economically important problem to address is the biofouling of ships, but biofouling is also a challenge for the maintenance of heat exchangers and desalination plants, as well as for fisheries and aquaculture. For the shipping industry, the main problem with biofouling is that the bodies and shells of the attached organisms increase the hydrodynamic drag of the vessel, leading to higher energy consumption and/or reduced performance. It has been estimated that an 86% increase in propulsion power is necessary for a vessel with heavy calcareous biofouling to maintain operations at high speed, compared with an equivalent vessel with a smooth coating. Such a power increase obviously leads to significantly higher fuel consumption and increased costs. In addition, ships with fouled hulls need to go into dry-dock to be cleaned, which adds to the cost of maintenance and leads to a loss in revenues due to operational downtime. The environmental impact of marine biofouling is also significant, due to the increased release of emissions, such as CO₂, SO₂ and NOₓ, associated with the higher fuel consumption. An additional concern is that the fouling organisms, which are inadvertently transported around the world, may act as invasive species, posing a direct threat to local ecosystems.

**Figure 2.1.**
A) Biofouling on the hull of a pleasure craft after one season in Swedish waters. The lower (unfouled) part of the hull had been painted with an antifouling paint. B) Close-up of the same hull, showing the shells of barnacles covering the area lacking a protective coating. Images kindly provided by Kristina Buchholt. C) Submarine with algal biofouling. Image kindly provided by James Callow.
2.1.1 Marine biofouling and its remedies – a historical outlook

Marine biofouling is likely to have been seen as a problem for thousands of years, long before any effective preventive methods were available. In Roman times, the author Plutarch recognized that a ship’s performance would become impaired if “weeds, ooze, and filth stick upon its sides”.\(^{[17]}\) It is thought that many of the surface treatments known from historical records (e.g. tar, wax and pitch coatings, and lead sheathings) were adopted to decrease the amount of fouling, though these measures may also have been intended to waterproof the ships or to protect them from shipworm.\(^{[18]}\) It was only upon the introduction of copper sheathing in the late 18\(^{th}\) century that a viable method to protect ship bottoms from biofouling became available. The technique, which meant that the surface of the hull was covered with copper plates, was originally applied for protection against shipworm. However, it soon became evident that the surface of the copper fouled less than conventional materials, and it was therefore used extensively until the introduction of iron hulls.\(^{[18]}\) Corrosion of iron components in contact with the copper had always been a problem with the copper sheathing technique, but iron hulls were even more vulnerable, and the risks of the technique were deemed to be too great.\(^{[1]}\) Hence, for a long period of time, no efficient antifouling treatments were available for iron ships, which despite this fact increased in popularity and largely replaced wooden ships. Great efforts to develop antifouling treatments were made in the 19\(^{th}\) century, but the approaches were generally thoroughly unscientific and did not lead anywhere.\(^{[1]}\) A few attempts were more successful; in particular those involving different types of organic coatings containing copper, arsenic or mercury compounds. This approach of using biocides dispersed within a paint matrix, which was intended to produce a slow and even leaching rate of the active compounds, was refined further over the years, and became the dominant antifouling technology in the early 20\(^{th}\) century.\(^{[18]}\)

In the 1950s the potent antifouling effects of organotin compounds were discovered, and this led to the rapid employment of tributyltin (TBT) as the main biocidal additive to antifouling coatings.\(^{[11]}\) The development of so-called self-polishing copolymer paints, with hydrolyzable methacrylate-conjugated TBT incorporated as a comonomer in the paint resin, was a further improvement. These TBT-containing coatings, often incorporating copper additives, were remarkably effective and were greeted as the definitive solution to the marine biofouling problem.\(^{[19]}\) However, it soon became clear that the effects of TBT reached far beyond the ship hulls, causing severe adverse effects on many marine ecosystems, in particular mollusk populations.\(^{[20]}\) As a consequence, the use of TBT for marine antifouling purposes was banned in many countries during the 1980s.\(^{[16]}\) In Sweden, the use of TBT paints was prohibited for vessels under 25 m in length in 1989, and the International Maritime Association has later imposed a total and universal ban on the application of TBT-containing paints (since 2003) and the use of vessels with such coatings (since 2008).\(^{[20]}\) TBT has therefore been replaced with other biocides with more environmentally benign effects, particularly copper compounds. However, copper also has adverse effects on the environment, which has led to it being banned for pleasure craft shorter than 12 m on the Swedish East Coast.\(^{[21]}\) In addition,
copper is not always effective and is often used together with other “booster” biocides to provide a more wide-spectrum effect.\textsuperscript{[21]} The ill-fated introduction of TBT and other persistent organic pollutants has increased the awareness towards the use of biocides in the marine environment in general, and other routes for achieving good antifouling properties are now actively sought.

Figure 2.2. Important biofouling organisms. A) Scanning electron microscope (SEM) image of \textit{Ulva linza} spores (see Figure 2.1 for \textit{Ulva} fouling). B) \textit{Semibalanus balanoides} barnacle cyprids. C) Adult settled barnacles. D) SEM image of a biofilm dominated by diatoms. Images A and D kindly provided by James Callow. Image C courtesy of Ashley Cottrell.

\subsection*{2.1.2 Modern approaches to reduce marine biofouling}

The following section will highlight the main approaches for achieving antifouling effects without the use of leaching biocides. That is, coatings with purely “physical” means of action, which affect only those organisms which attempt to settle or have settled on the treated surface. Other approaches based on novel biocides are also likely to become important in the future, but they will not be further discussed here.

Silicone-based coatings represent the most prominent non-biocidal antifouling approach in use today. These coatings do not necessarily resist the settlement of sessile organisms, but they provide a very poor substrate for persistent attachment, in particular for barnacles. If the shear rate becomes high enough (which will happen at relatively high speeds) the settled organisms may simply detach. The fouling-release effect appears to be due to a combination of mechanical softness,\textsuperscript{[22]} low roughness, low surface free energy,\textsuperscript{[23]} and a constant presence...
of low-molecular weight silicone oils at the interface.\[24\] Although effective under many
conditions,\[25\] the speed requirement (in excess of 20 knots in some cases) is the main
challenge which needs to be overcome before fouling-release coatings can be put in
widespread use.\[11\] In addition, the mechanical softness of the silicone-based coatings makes
them sensitive to abrasion, and the coatings are difficult to mend once scratched. Finally, the
presently available fouling-release coatings are not at all effective against diatom slime, which
therefore tends to dominate on the surface.\[26\]

Other chemistries, particularly fluoropolymers, have also been developed for foul release
applications.\[27\] However, these generally lack the softness believed to be important for the
good fouling-release properties of silicones. As a response to this concern, elastomeric
fluoropolymers have been developed and evaluated in lab-based biofouling assays, with good
results.\[28, 29\]

Another approach is to discourage the fouling organisms to adhere in the first place. This
“antifouling” approach may, in theory at least, be achieved by preventing the biological glues
excreted by the organisms to stick to the surface in the settlement phase. The available
chemical options to achieve such an effect will be reviewed later in this chapter. This was the
approach investigated in Paper II, and that and other studies show that this is a viable route to
decrease the settlement rate of a wide array of organisms.\[30-34\] One problem might be the
relatively poor long-term stability of conventional nonfouling chemistries, although efforts are
being made to design more durable alternatives.\[35, 36\]

Some of the most recently developed coating designs combine elements of both approaches,
by using amphiphilic polymers with both hydrophilic and hydrophobic segments.\[37-40\] The
idea behind this approach is that the coating should present a nanoscopically heterogeneous
surface, providing both foul release and antifouling features. This new approach appears to be
promising, and is presently being pursued by several research groups.

A somewhat related route to achieve antifouling/fouling release properties is to create micro-
scale patterns of structural features. The scale and geometry of the microstructures are critical,
since increased roughness typically leads to more fouling, rather than less. Only very specific
patterns appear to have a positive effect,\[41, 42\] and then generally only for specific organisms,
for which the size scales of the patterns are optimized.\[43\] Therefore, structured surfaces with
several overlaid patterns with different size scales may be more successful,\[44\] but the problem
of applying them over large surfaces still remains to be solved.

Most of the approaches above are still at the experimental stage, and are rather far from being
used in real applications. In fact, the problem of marine biofouling has only recently received
widespread attention from the academic community. In the last few years, concentrated
efforts have been made to investigate novel routes for creating non-toxic, yet effective
coatings for biofouling prevention. Many of the studies cited above are results of these efforts.
In Europe, the AMBIO integrated project (Advanced Nanostructured Surfaces for the Control
of Biofouling),\[45\] funded by the European Commission, has provided an interdisciplinary
research infrastructure for advanced marine biofouling studies. In North America, similar research is funded by the US Office of Naval Research (ONR). No doubt, further research efforts will be needed before a new and sustainable solution to the problem of marine biofouling is found, but many of the recent results are encouraging.

2.2 Biomedical applications

The use of synthetic materials in biomedical applications may seem completely unrelated to the construction and maintenance of ships, but there are in fact some very clear similarities between marine biofouling and biomaterials science. An obvious example is the formation of microbial biofilms, which is a challenging issue for both fields. Another intriguing connection was discussed in a recent research paper which suggested that two of the most important biological mechanisms in each respective field, namely barnacle cement polymerization and blood coagulation, are evolutionary related. The two fields certainly share a common need for understanding complex biomacromolecular adsorption and adhesion processes in high-ionic strength aqueous environments. The traditional method to avoid marine biofouling – the application of a relatively thin coating that protects and covers a bulk material with unfavorable surface properties, possibly while leaching an active substance – has become increasingly important for biomaterials applications. In addition, it seems that some of the approaches to develop nonfouling surfaces for biomedical applications could, at least in theory, be directly applicable also for the prevention of marine biofouling. This close relationship is the reason for dealing with aspects of both subject areas in this work. However, the field of biomaterials science is vast, and therefore two aspects of relevance to the work performed have been selected to be briefly described here; blood-contacting biomaterials applications and biosensor applications.

2.2.1 Biomaterials in blood-contacting applications

The human body is well-protected against excessive blood losses through the hemostatic system, which regulates the delicate balance between the need for blood clotting and for unobstructed blood flow. Immediately after an injury, a complex physiological response acting to stem the blood flow locally and repair the damage is initiated. However, this response, which has evolved over millions of years to provide adequate protection against the inevitable injuries of everyday life, is not well-equipped to handle exposure to synthetic materials introduced in the blood stream. Nevertheless, such materials, in the form of prosthetic devices, are used on a large scale for repairing or alleviating injuries and defects which cannot be healed naturally. Examples are artificial heart valves, coronary stents and vascular grafts. Many of the materials used in these applications are relatively primitive, and have been in use for decades, during which they have proven their functionality and ability to perform with acceptable safety. This does not mean that they are truly blood compatible, however: The risk of thrombotic events (formation of blood clots) is always
present, and anticoagulant drugs, which increase the risk of bleeding,[52] must in many cases be taken continuously after surgery. Despite massive efforts to improve these materials, very few genuine advances have been made over the years, making this question seem at least as difficult to solve as that of marine biofouling.

The complexity of the problem is probably at the root of the poor success rate for modern approaches to fabricate blood compatible materials. Blood plasma coagulation, which involves several protein cascades, can be initiated in a number of ways, some of which are not completely elucidated.[53] Cells, in particular platelets[54] and leucocytes, play an important part in modulating the hemostatic and immunologic host response. The complement system, which has evolved to handle infectious microorganisms and other foreign elements, reacts strongly upon contact with many artificial surfaces. All these effects are linked and take place simultaneously, under flow conditions.[55] Due to these complexities, it is not known exactly how a potentially successful artificial biomaterial surface for blood contacting applications should be designed, or if such a surface even can be designed.[51] One persistent idea is that adsorption/adhesion of blood components, in particular plasma proteins and platelets, are crucial for the subsequent events, whether it be implant failure or healing.[56, 57] It should, however, be noted that this response not necessarily will manifest itself in the form of protein adsorption – even a material which does not retain much protein on its surface might interact unfavorably with blood, creating thrombotic events downstream from the device.[58] Whatever the outcome, the physical and chemical properties of the synthetic surface are determining factors for the response. Coatings are in this context a convenient way to exert control of surface properties, since they can be applied to bulk materials with reliable and well-proven mechanical properties. A common approach to the design of prospective blood compatible synthetic coatings is to try to suppress all interactions with proteins.[59-61] This “stealth” approach has been shown to be a successful method for prolonging the blood circulation half life of drug-containing nanoparticles,[62] but the results have so far been limited for prosthetic devices.[55] Another option is to design the coating so that “benign” proteins, such as serum albumin, are selectively adsorbed, with minimal conformational changes.[63-66] Coatings containing anticoagulant drugs such as heparin have also been developed[67-70] and used with some success in clinical applications.[71] It remains an open question whether any of these methods will be used to successfully render synthetic materials completely blood compatible in the future. Even if this goal could be reached, other issues, such as fibrous encapsulation, must also be dealt with before a biomaterial can be confidently said to be free of complications.[50]

2.2.2 Bioanalytical devices

Bioanalytical devices, such as biosensors, protein microarrays, and disposable diagnostic biochips also need to be capable of handling protein – surface interactions. However, although the investigated sample may well be in the form of a few drops of blood, there are far fewer critical issues to consider for the development of surface materials for bioanalytical devices, compared with in vivo blood contacting materials. First of all, the host response becomes
relatively unimportant since the device does not normally need to be introduced into the
human body and is typically only exposed for a short time to a small volume of biofluids,
which will in any case be discarded. Secondly, it is possible to manipulate the sample before
it is exposed to the surface. Typical examples are the use of plasma or serum instead of whole
blood, and the addition of chelating agents or heparin to inhibit the coagulation cascade.
Finally, although the readout of the device may be critical for a correct diagnosis, a possible
failure will not normally be directly life-threatening. The latter statement is the reason for the
relatively moderate regulatory demands imposed on devices of this type. Despite these reliefs,
the designer of a novel bio-analytical device still has a few major surface-related challenges
which need to be overcome, mainly relating to protein adsorption effects.

The detection principle of one of the most common classes of bioanalytical devices is based
on surface-immobilized antibodies or other recognition elements which selectively bind to an
antigen or ligand (generally a protein) in the sample, in turn leading to a detectable response.
It is important that a high concentration of active antibody can be immobilized to the surface,
as this increases the potential sensitivity of the assay. The most widely used technique for
immobilizing proteins for this application is to utilize physical adsorption onto hydrophobic
or positively charged surfaces (Figure 2.3 A). This is convenient, but leads to a few
potential drawbacks. Flat two-dimensional sensor surfaces can never support more than one
monolayer of immobilized protein. If the immobilization method in addition induces
denaturation of the adsorbed proteins – as is the case for hydrophobic polystyrene – the
effective concentration of functional recognition elements on the surface can become quite
low. In order to optimize the immobilization strategy, both a higher total concentration of
immobilized proteins and a higher degree of biological activity is desirable. The latter can be
achieved by using a material which does not induce conformational changes of the protein,
and/or by directing the protein so that the binding epitopes are available for interactions with
the sample solution (Figure 2.3 B and C). A high concentration of protein can be
achieved by increasing the effective surface area, e.g. by using particles or a porous (three-
dimensional) matrix instead of a flat surface. Chemical coupling is used to immobilize the
protein in these cases. A successful example of the latter approach is the carboxylated dextran
matrix typically used for surface plasmon resonance-based biospecific interaction analysis.

Another issue of major importance to biosensor applications is the non-specific adsorption of
proteins. The effects on the biosensor response depend on the detection principle. If “label-
free” methods are used, for example surface plasmon resonance, quartz crystal microbalance,
or ellipsometry, the detected quantity will simply be proportional to the adsorbed mass, and
no distinction can be made regarding the type of protein bound to the surface (Figure 2.3 E).
Therefore, non-specifically adsorbed proteins may lead to false positive responses and a poor
signal-to-noise ratio. This may be partly avoided in “labeled” methods, typically employing
fluorescently labeled secondary antibodies which selectively recognize the analyte, after it has
been captured on the surface by the immobilized antibodies (Figure 2.3 D). Other adsorbed
proteins from the sample will therefore not directly influence the response. However, non-
specific adsorption of the secondary antibody must be avoided in this case. The strategies for reducing non-specific protein adsorption commonly involve a “blocking” step wherein the surface is exposed to a high-concentration protein solution. The blocking protein is intended to adsorb to any available empty surface sites, thereby restricting further nonspecific adsorption of other proteins. Bovine serum albumin and casein are commonly used in this function.[73] However, since blocking does not guarantee complete resistance to non-specific adsorption[77] and since it adds an additional step to the preparations, it should be avoided if possible.

Figure 2.3. A-C): Different routes for immobilization of recognition elements (e.g. antibodies, Y-shaped in figure) for the detection of a protein analyte (gray, roundish). A) Physical adsorption, which may induce conformational changes. B) Random chemical immobilization to a three-dimensional polymeric brush-like matrix. C) Directed immobilization to a three-dimensional matrix. D-E): Different detection routes. D) “Labeled” method; fluorescently labeled secondary antibody. E) “Label-free” method, which directly detects analyte binding (e.g. surface plasmon resonance).

The ideal biosensor surface should, according to the demands stated above, allow high-density immobilization of antibodies or other biological elements required for detection, while resisting non-specific protein adsorption. Although neither of these requirements are strictly necessary (as evidenced by the wide-ranging use of polystyrene surfaces for similar applications), both of them will potentially increase the sensitivity and selectivity of the device. An attempt to develop a three-dimensional, protein resistant surface chemistry for biosensor applications was the starting point for the work described in this thesis, as described in Paper I and in the subsequent work by Larsson et al.[78-80]

In many cases, the active biosensor surface itself represents only a fraction of the total surface area in contact with the biological sample. Integrated biosensor chips with microfluidic flow systems may contain intricate liquid handling systems, filters and valves. The additional surfaces may need to exhibit certain properties, such as high or low hydrophilicity, to provide the intended function. It is desirable that these surfaces are also designed so that protein adsorption can be suppressed throughout the system, to avoid depletion of the sample before it arrives to the sensor surface. Paper IV shows how surface chemistries with different properties may be fabricated and tested and Paper V is a description of a potential surface chemistry for use in a demanding blood contacting application, namely platelet function analysis.
2.3 Strategies for designing nonfouling coatings

As has already been indicated in the previous sections of this chapter, the nature and amount of surface-adsorbed proteins are crucial factors determining the biological response to an artificial surface. According to a somewhat simplified view, the outcome of an encounter between a solid surface and a protein may be one of three cases [81] (illustrated in Figure 2.4):

1) The protein does not adsorb to the surface, and stays dissolved.
2) The protein adsorbs reversibly to the surface.
3) The protein adsorbs irreversibly to the surface, with conformational changes. Several forces, with magnitudes and signs depending on the properties of the protein, the sorbent surface, and the solution, are involved in this process. The total change in the free energy of the system determines the outcome. [82] The adsorption of proteins to hydrophobic surfaces is largely driven by the entropic gain made when water molecules no longer need to be in contact with the hydrophobic surface, making it possible for them to arrange in a more energetically favorable manner. [83] In addition, van der Waals forces and rearrangements within the protein structure contribute to adsorption. [84] A globular protein can be described as having a densely packed hydrophobic core surrounded by a hydrophilic coat of polar amino acids. If the densely packed core can become somehow less organized, which may be the result of adsorption, an entropy gain can be made. This denaturation process normally leads to irreversible adsorption. On hydrophilic surfaces, no significant entropic gain can be made by displacing water molecules, since they are already in an energetically favorable order at the surface. Instead, adsorption is mainly determined by electrostatics [85] and hydrogen bonding between the polar protein “shell” and the surface, together with possible structural rearrangements in the protein. [82, 84] Although most protein-surface combinations tend to lead to irreversible adsorption, reversible adsorption may take place at hydrophilic surfaces, if the attractive and repulsive interactions are in reasonable balance. [82] In some cases, the protein does not adsorb to the surface at all. The most common case is when there is electrostatic repulsion between a charged and rigid protein (which will not unfold upon adsorption) and a hydrophilic surface with the same charge. [82] It can therefore be relatively easy to design a surface which rejects adsorption of one particular protein. However, since complex biofluids contain proteins with widely varying charges and structures, it is significantly more difficult to design a surface so that all proteins are rejected.

Figure 2.4. A simplified view of protein adsorption, with three possible outcomes. A) Protein rejection. B) Reversible protein adsorption. C) Irreversible protein adsorption with conformational changes in the protein.
A truly protein resistant synthetic material, with a surface that will resist adsorption of all proteins even during exposure to complex biological fluids for extended time periods, is yet to be invented. The difficulties are particularly obvious in the field of marine biofouling, where the surface will face protein systems which have evolved over hundreds of millions of years to form highly efficient and versatile adhesion mechanisms. Nevertheless, surfaces displaying very low protein adsorption do exist, and several different viable chemical approaches for the minimization of protein adsorption have been demonstrated. To this end, a few general design principles for the minimization of protein adsorption have been empirically defined, on the basis of extensive protein adsorption studies of many different materials. According to these results, the ideal surface should be hydrophilic, contain functional groups with hydrogen bond acceptors but without hydrogen bond donors, and be electrostatically neutral. However, exceptions exist even to these rules, at least regarding the hydrogen bond donors. The sections below will describe the most thoroughly investigated alternatives for achieving protein resistant materials.

2.3.1 Poly(ethylene glycol)

Poly(ethylene glycol) (PEG), which is also known under the names poly(ethylene oxide) (PEO), polyoxyethylene or polyoxirane, is a water-soluble polymer with very special properties. The chemical structure of this polymer is shown in Figure 2.5. It is widely used as a component of nonionic surfactants and was in the early 1980s found to afford very high resistance to protein adsorption when grafted to surfaces. It has ever since been the primary polymer of choice for applications where high protein resistance is required, and has repeatedly been shown to promote ultralow protein adsorption from complex biofluids. In addition, it has extremely low toxicity (although ingestion should be avoided) and is therefore attractive for uses in biomedical applications.

The exact reasons for the protein resistance of PEG surfaces have been studied and discussed extensively and are still subject to some uncertainty. It was originally thought that the effect mainly relates to steric repulsion from the large excluded volume of the mobile PEG chains, resulting in energetically unfavorable compression of the polymer segments upon approach of a protein (Figure 2.6). However, other hydrophilic polymers, which superficially show...
similar swelling properties to PEG, do not produce the same effect.\[91\] Therefore, other features unique to the PEG chemistry are likely to also play a part in the protein resistance. In fact, several factors in combination appear to be responsible for the optimal protein resistance of PEG. First of all, it does not hold a formal electrostatic charge (although it has been reported to attract hydroxide ions from solution\[92\]). This ensures that no electrostatic attraction of proteins takes place. Secondly, the most energetically favorable conformation of PEG at low temperatures (a gauche conformation about the C-C bond\[93, 88\] see Figure 2.5) has a large dipole moment. This makes the PEG chains polar and enables the oxygens to act as hydrogen bond acceptors and interact strongly with the surrounding water molecules. The energetically favorable water structure around the PEG molecule is believed to play a major part in the control of protein adsorption (Figure 2.6 B). An interesting note is that neither poly(methylene glycol) nor poly(propylene glycol) are particularly water soluble, or protein resistant – PEG is clearly a unique polymer.\[94\]

The many experiments carried out with well-controlled PEG surface chemistries, particularly in the form of self-assembled monolayers, have provided indications as to the most important properties of protein resistant PEG coatings. It was early established that the average length of the PEG chains influences the protein adsorption of PEG-coated materials.\[89, 95\] The general trend is that longer PEG chains give higher protein resistance, in particular in demanding applications.\[96\] However, also oligo(ethylene glycols) (OEG) with only two to three EG units have been shown to be protein resistant under many conditions.\[31, 95, 97\] This effect, which clearly cannot be due to steric repulsion, instead appears to relate to the formation of a strongly bound hydration layer on the OEG surface,\[98\] as discussed above. Recent neutron reflectivity experiments have revealed that protein solutions become strongly depleted of proteins in the region immediately (a few nm) next to OEG SAMs of this type.\[81\] It is most likely that the protein adsorption characteristics also of longer PEG chains depend greatly on this effect, along with contributions from steric repulsion. The idea that chain length is critical for the protein resistance of PEG has therefore been revised with time. The other main issue to consider is the density of grafted PEG chains. If the density is too low, proteins may adsorb to the underlying material between the PEG chains.\[99\] If the density is too high, as can be the case for highly ordered self-assembled monolayers of OEG prepared on silver,\[100\] protein

![Figure 2.6. Cartoon illustrating the two main explanations for the protein resistance of PEG. A) The protein (gray) is rejected due to steric effects in long PEG chains. B) Water molecules form an energetically favorable hydration shell around the (short) PEG chains, which the protein cannot disrupt. Not drawn to scale.](image-url)
adsorption may also take place. This effect appears to be related to the unusual conformation adopted by the crowded OEG chains in this particular case. Very dense layers of grafted long-chained PEG have also been shown to display increased protein adsorption compared with layers prepared with intermediate densities.\cite{101-103} Such dense end-grafted layers can only be prepared by raising the solution temperature and/or adding certain salts during the preparation phase.\cite{104} This is due to the peculiar solution behavior of PEG in water – the solubility decreases with temperature.\cite{88} If the PEG chains are securely tethered to a surface in this state, in which much of the hydration layer is lost, the high-density conformation appears to persist as the temperature is lowered, yielding higher protein adsorption. Although it is not impossible that this transition (referred to as the cloud point) has implications on protein adsorption also for less dense PEG layers under physiological conditions,\cite{105} it should not be particularly relevant as it comes to effect at relatively high temperatures (>50 °C for PEG homopolymers).\cite{106} Finally, the terminal group of the PEG chain may be of importance for OEG monolayers and other short PEG chains for which a high density of untethered chain ends will come in contact with the protein-containing solution. The terminal group has also been implicated to affect the protein adsorption properties of long, but very densely grafted, PEG chains.\cite{103, 107} It is often found in these cases that hydrophobic terminal groups (e.g. -OCH₃) lead to higher protein adsorption than hydrophilic groups, such as hydroxyls.\cite{31, 108} However, studies in which no such effect was observed have also been presented.\cite{86}

The Achilles heel of PEG is probably its poor stability. The polymer readily undergoes oxidative degradation, especially at elevated temperatures.\cite{109} A range of bacteria can also metabolize PEG chains, primarily with the help of alcohol dehydrogenase enzymes.\cite{110, 111} Long-term studies have repeatedly shown that PEG coatings fail to stay protein resistant over extended periods of time,\cite{112-116} although other reasons for failure than PEG degradation can be suspected in some of these cases. Question marks have therefore been raised regarding the viability of using PEG as a long-term protein resistant material and the lack of clinical success for PEG-based biomaterials\cite{55} appear to support this notion.

### 2.3.2 Zwitterionic materials

A zwitterionic moiety contains formal positive and negative charges, but holds a zero net charge. Many amphoteric molecules, such as amino acids, are zwitterions at the appropriate pH, when both the carboxylic acid and amine are in the charged state. They are known to display excellent dermatological properties, and are therefore used in specialty surfactants for soaps and shampoos.\cite{88} The most commonly used zwitterionic moieties are shown in Figure 2.7. The cationic group is typically a quaternary ammonium while the anionic group may be a carboxylate, sulfonate, or phosphate. The fact that the net charge is zero is of great importance to zwitterion – protein interactions since, in analogy with PEG, the lack of a net charge ensures that no electrostatic attraction of proteins will take place. Of course, a lack of charges alone is not enough to make a surface protein resistant. However, the charged groups are strongly hydrated, and the water is not easily displaced, which leads to a resulting repulsive interaction with proteins. In contrast with PEG, examples of protein resistant zwitterionic
surfaces can be found in nature, where the most famous example is the cell membrane of red blood cells, which is lined with zwitterionic phospholipids. The biomimetic approach of trying to emulate this system by synthesizing polymers with phosphorylcholine moieties has been relatively successful in biomaterials applications, both for bulk materials and coatings. In addition, other zwitterionic groups, such as carboxybetaines and sulfobetaines, have also been shown to afford extremely low protein adsorption, which opens up further possibilities for using zwitterionic materials in biomedical and biofouling applications. Carboxybetaines are particularly interesting since they contain a terminal carboxylic acid, which is a convenient handle for conjugation of biomolecules. Polymers containing carboxybetaines have therefore been suggested as ideal materials for surface coatings for bioanalytical devices in blood contact.

![Figure 2.7. Zwitterionic/charge-balanced groups. A) Carboxybetaine. B) Sulfobetaine. C) Phosphorylcholine. D) Example of an ammonium/sulfonate ion monomer pair copolymerized at 1:1 ratio. E) Methacrylate structure (the zwitterionic group is substituted at the position of the X).](image)

Another exciting opportunity lies in charge-balanced materials, which are not zwitterionic per se, but contains both positive and negative charges in equal numbers. They therefore display very similar properties to zwitterionic materials in terms of protein adsorption. The concept has been demonstrated for self-assembled monolayers made up of mixed thiols with positively and negatively charged terminal groups. More recently, copolymers based on the same concept have been synthesized from differently charged monomers, both as coatings and as bulk materials. They have been demonstrated to be highly protein resistant, provided that the charge is truly balanced. While balancing the charge can be a challenge, this extra optimization work is likely to be compensated by the fact that the individual monomers have less complex chemical structures than the zwitterionic alternative, making them less costly to synthesize. The work described in Paper III in this thesis shows a related method to create charge-balanced materials with high protein resistance, using a gradient approach with laterally varying charge. At the position of charge balance the material becomes protein resistant, while large amounts of protein are adsorbed in the respective cationic and anionic areas.
In conclusion, zwitterionic and charge-balanced materials appear to be more versatile alternatives to PEG for achieving protein resistant coatings. The short-term performance appears to be similar, while the long-term performance of the most recently developed zwitterionic coatings remains to be thoroughly examined. However, at least phosphorylcholine-based polymers appear to have performed well in extended human trials and are currently used as coatings for drug-eluting stents.\cite{131, 132} PEG is known to have stability problems, which are as inherent to the polymer structure as the high protein resistance, while a range of possible molecular configurations may be imagined for zwitterionic/charge-balanced coatings. Therefore, these coatings could potentially become a very useful complement to PEGs for applications when low non-specific protein adsorption is desired.

2.3.3 Other nonfouling chemistries

Although a huge number of different materials have been evaluated for their protein resistance, only a few in addition to PEGs and zwitterionic materials have been shown to completely prevent protein adsorption. Many of these have, in analogy with phosphorylcholine, been developed using a biomimetic approach. The following section outlines two of these strategies.

Carbohydrates. The surfaces of many cell types are covered with a polysaccharide or glycoprotein matrix, believed to play a fundamental role for keeping the cell surface free from adsorption of biomacromolecules. This layer, referred to as glycocalyx, has been used as an example for preparing protein resistant coatings.\cite{133} Dextran, a glucose-based polysaccharide (Figure 2.8 A), was mentioned in the previous section as a successful coating for biosensor surfaces. A number of studies have focused on biomaterials applications of dextran, and many different methods to prepare dextran-containing coatings have been devised.\cite{134-136} The best of these appear to suppress protein adsorption more or less completely. Since dextran is rapidly degraded in the presence of microorganisms, the stability of these coatings can be questioned. Recent results indicate that the long term-stability of at least one type of dextran-based coating is poor in a bacterium-enriched environment,\cite{137} which implies that any future success in biofouling applications is unlikely. Another important aspect of these materials was highlighted in a recent study by Cao et al.,\cite{138} who showed that coatings composed of surface-tethered alginic acid, hyaluronic acid, and pectic acid were relatively resistant to cell adhesion and protein adsorption as long as they were not exposed to divalent cations, particularly calcium. Complexation of calcium by the carboxylic acids in the polysaccharides made the coatings less protein resistant. Since seawater has a relatively high concentration of calcium the coatings would always be in a “non-resistant” state if used in a marine biofouling application, which was the initially intended application in this work. Consequently, the effects of the medium in which the protein (or cell, or organism) is suspended must be taken into account, when developing protein resistant coatings for use in “real life” systems. Another issue to consider for carbohydrate-based chemistries is the risk/possibility of specific interactions between the naturally derived coating material and biological species. Heparin,
which was discussed above, is actually a striking example of a polysaccharide with potent biological reactivity. It should also be noted that a range of proteins, so-called lectins, are adapted to specifically bind to carbohydrates.\[^{139}\]

Not only the macromolecular versions of the carbohydrates (e.g. dextran), but also the monomers and dimers have proven to afford protein resistance to coated surfaces. Prime and Whitesides showed that thiol SAMs with a terminal maltose group were relatively protein resistant, although they were clearly less efficient than their OEG counterparts.\[^{140}\] Similar films presenting mannitol groups have been shown to be comparable with OEG SAMs in preventing protein adsorption, and to be superior for long-term prevention of cell adhesion.\[^{141}\] Hederos et al. used an approach in which galactosyl, a monosaccharide, was partly methylated to produce a highly protein resistant surface.\[^{142}\] The route of partly modifying a the carbohydrate might, in addition to potentially making it more protein resistant, also be a way to decrease the risk of biological recognition or rapid enzymatic degradation\[^{143}\] in a biological environment.

![Figure 2.8. Nonfouling alternatives to PEG and zwitterions. A) Dextran. B) A normal peptide chain, R indicates side chain. C) Peptide‐like polymer type I: Used by Statz et al.\[^{145}\] Polymers with other side chains than the methoxy‐terminated version shown here may also be used. D) Peptide‐like polymer II: poly(2‐methyl‐2‐oxazoline) used by Konradi et al.\[^{146}\] Peptides and peptide‐like polymers. Normal polypeptides (or, indeed, surface‐adsorbed proteins) may, if properly designed/selected, exhibit high protein resistance.\[^{144}\] However, the peptides (structure shown in Figure 2.8 B) suffer from the same stability problems as most other naturally derived materials. To resolve this, techniques for synthesizing peptide analogues with somewhat modified chemical structure have been developed for the fabrication of protein resistant coatings. Two types of nonfouling peptide‐like polymers have been described to date (Figure 2.8 C and D).\[^{145, 146}\] Both of them have a side chain attached to the nitrogen atom instead of the Cα carbon. This has two important implications: The hydrogen bond donating capability of the backbone is lost, and the ability of enzymes (proteases) to degrade the material is diminished. The design possibilities for these new types of chemistry are immense, due to the large number of possible side chain configurations. The
most effective version to date, in terms of protein resistance, seems to be a peptide-like polymer with methoxy-terminated side chains. Coatings based on this chemistry have shown excellent long-term resistance to cell adhesion\textsuperscript{[145]} as well as to bacterial adhesion.\textsuperscript{[35]}

2.4 Testing methods

Studies intended to test how artificial materials interact with their biological environment have often been limited to the study of adsorption of isolated biomacromolecules onto the material surface.\textsuperscript{[56, 86, 95, 140]} It could be argued that one reason for the popularity of this approach is that it is relatively easy to perform such studies; in principle only the surface of interest, a protein solution and access to a suitable characterization technique are needed. An abundance of proteins are available for testing, and surface chemistries can be prepared with immense variation, leading to an almost infinite amount of possible combinations, of which most would simply constitute a grave simplification of reality with little actual biological relevance. However, the rationale for the test concept is based on a sound idea – namely, that all biological interactions with artificial surfaces start with the adsorption of biomacromolecules, which in turn mediate further events.\textsuperscript{[50, 147]} The challenge lies in devising the test so that adsorption of the right proteins under the right circumstances is investigated. Some examples are well-known and thoroughly studied, such as the adsorption of the blood plasma protein fibrinogen, which leads to platelet adhesion.\textsuperscript{[148]} Protein adsorption studies based on more complex protein solutions, such as whole blood, plasma or serum,\textsuperscript{[149]} may be a further step towards understanding the protein-material interactions. If properly designed, relatively simple protein adsorption studies can, in fact, give important clues regarding the properties and performance of a synthetic material in a biological environment. Several protein adsorption studies have been carried out in this work, for instance in Paper I, Paper II, Paper III, Paper IV, Paper V and Paper VIII. The more complex coagulation experiments in Paper IV are a further step towards the final application. However, more complex testing procedures, involving first animal studies and later human subjects, are required before a biomaterial can be used in patients. The economic cost and regulatory requirements of such an enterprise can be very challenging hurdles to overcome, however.\textsuperscript{[51]}

For tests of potential marine biofouling-resistant materials, the picture is quite different. The adhesion mechanisms of sessile marine organisms are at least as complex as their biomedical counterparts, and much less well understood. The actual biomolecules responsible for adhesion are generally not known, let alone isolated and available for adsorption experiments (although a few exceptions exist). The testing protocols have traditionally been field tests, which are very hands-on and similar to the intended end-use. Originally,\textsuperscript{[1]} the method for testing antifouling coatings seems to roughly have followed these steps: 1) Find a suitable ship. 2) Persuade the captain to let you put your coating on his ship (or even better – sell it to him for a profit). 3) Apply the coating, let the ship sail. 4) Wait for the result. 5) Try to avoid the captain. In later times, submerged panels with applied coatings have been introduced as a

\begin{center}
\textbf{Paper viii}
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more convenient alternative (Figure 2.9 A). Tests of this type are appealing since the results are (superficially) easily interpreted and allow the evaluator to discard those alternatives that will clearly never work. A major challenge is the poor control of many critical test parameters. For instance, the biofouling pressure varies greatly over the seasons, both in species and intensity, and is highly weather dependent. For this reason, tests that are aimed to reduce the complexity and the number of unknown variables have become increasingly important. These tests are often performed using selected organisms in a laboratory environment under controlled conditions (Figure 2.9 B, C). Such studies may, of course, also be used to learn more about the behavior of the organisms involved, possibly giving new clues for antifouling strategies. The progress in this field has been achieved by persistent efforts by a small number of labs worldwide. We have cooperated with some of these groups, notably the Callow lab in Birmingham, UK, who collect algal spores of the species *Ulva linza* and also work with diatoms, and the Clare lab in Newcastle, UK, who work with barnacles. The results of these experiments may be seen in Paper II. We have also, together with the group in Newcastle, developed a new test protocol for barnacle biofouling, drawing on experiences from the bioanalytical field. The method involves determining the surface adsorption of a specific settlement-related protein and the use of imaging surface plasmon resonance for studying the behavior of the barnacle larvae, so-called cyprids (Papers VII and VIII). This method may be compared to that described by Heydt et al, who used in-line holography to study the behavior of *Ulva linza* spores. The challenge in devising “artificial” lab tests of this type lies in determining the viability of the test, both in terms of the extent to which conclusions can be drawn regarding the natural behavior of the organisms, and whether the ultimate performance of the coating in the marine environment can be predicted. As an example, the hydrogel coatings which showed very promising results in the lab-based assays described in Paper II, did not perform particularly well in later field tests, although the exact reasons for this could not be deduced from the tests. However, detailed and controlled studies such as these are definitely necessary to provide a foundation for the rational design of non-toxic coatings for the prevention of marine biofouling.
Figure 2.9. Strategies for evaluating the biofouling performance of novel coatings. A) Coated steel test panels after immersion in the Atlantic Ocean off the coast of Florida, USA. B) Coated microscope slides used for lab-based assays, as in the AMBIO project. C) Rotating immersible drum for release tests, as used at the TNO site in Den Helder, the Netherlands. The mounted slides are encircled for clarity.
3 FABRICATION OF SURFACE-GRAFTED COATINGS

The main objective of this work has been the development of ultrathin hydrogel coatings for the reduction of adsorption of biomacromolecules onto surfaces, in turn enabling uses in advanced biomedical and biofouling applications. In order to put this work into context, a description of some of the most important methods to achieve similar goals are described below. Technically important conventional coating deposition techniques such as painting, spraying, casting etc. have been excluded from this review due to their limited scientific novelty. However, it should be noted that methods such as these most definitely will be required for large-scale application of antifouling coatings, for instance on ship hulls.

A great variety of different methods to prepare surface-bound polymer coatings have been developed over the years.\cite{4, 152} In the literature, these methods are often classified according to the preparation technique; specifically, whether the polymer chains are first synthesized and then tethered to the surface or if the polymerization reaction propagates directly from the surface. The terms “grafting to” and “grafting from” are used to describe these two cases, respectively\cite{4} (Figure 3.1). The latter term may be used synonymously with the term “surface-initiated polymerization”. Alternatively, the methods have traditionally been divided according to the manner of attachment to the surface, which is either based on physical forces (i.e., the coating is physisorbed), or covalent in nature (chemisorbed).\cite{91} In the description below, this division has only been applied to the “grafting to” methods, as it appears to be relatively unusual for “grafting from” methods to be based on physisorption. Whenever this is the case, however, it will be mentioned in the text.

Both “grafting to” and “grafting from” methods have been applied successfully to prepare nonfouling coatings, although with major variations in performance and suitability for different applications. Versions based on the “grafting from” approach have developed most rapidly in the last decade due to recent advancements in controlled polymerization reactions.
This has allowed great control of many crucial parameters, including grafting density, polymer chain molecular weight and polymer composition. Below are some examples of the most widely used approaches for preparing surface-bound polymers, focusing on those of relevance for this thesis, and on recent developments within this field. A few synthesis techniques contain elements of both “grafting from” and “grafting to” methods, or do not in some other way adhere completely to the definitions stated above. However, for the cause of simplicity these techniques have been placed in the most appropriate sections, with any deviations from the norm noted.

3.1 Coatings prepared by “grafting to” methods

3.1.1 Physisorbed coatings
Polymers dissolved even in good solvents may adsorb spontaneously to solid surfaces in thermodynamically driven self-assembly processes. Such processes require intermolecular physical forces, for instance electrostatic or hydrophobic interactions in aqueous environments. The resulting non-covalently bound assemblies can be quite stable despite the relative weakness of the individual monomer-surface interactions, due to the large total number of interactions which need to be interrupted simultaneously for the polymer to desorb.\(^{[88]}\) This relatively simple principle has been utilized for preparing a great number of different coatings consisting of so-called polyelectrolyte multilayers (PEMs), fabricated by alternating layer-by-layer surface adsorption of cationic and anionic polymers from solution.\(^{[153]}\) Although they are in principle suitable for biological applications, the nonfouling applications for coatings of this type are limited by the fact that the resulting upper surface normally holds an electrostatic charge, which in turn tends to lead to high protein adsorption.\(^{[154]}\) However, it is possible to produce PEMs which display protein resistance by “capping” them with an additional layer of, for instance, zwitterionic\(^{[155]}\) or PEG-containing\(^{[156]}\) polymer.

![Figure 3.2](image.png)

**Figure 3.2.** Two common methods to graft polymers to substrates by physical adsorption. The gray segments symbolize non-fouling polymer chains. A) Positively charged polymer on a negatively charged surface. B) Block copolymer with hydrophilic and hydrophobic blocks.
A simpler and more common approach to create nonfouling materials is to omit the PEM construction procedure altogether and fabricate a coating consisting of a single, physisorbed nonfouling polymer layer directly on a suitable substrate. This technique requires that the adsorbing polymer contains both surface-binding “anchor” segments and free “buoy” segments, with a suitable ratio and intramolecular distribution. Polymers which comply with these demands can be formed by combining different types of polymer chains within a single macromolecule. Methods for synthesizing well-defined block copolymers have been known for more than a half-century, facilitating the early advances of the physisorption route of the “grafting to” approach. Another route to accomplish similar results is to prepare comblike graft copolymers with non-surface-binding chains grafted to a surface-binding polymer backbone. For applications in biologically relevant systems, the “buoy” section most often consist of a water-soluble polymer with no net charge, frequently PEG, but in some cases dextran or a peptide-like polymer. The “anchor” part is generally either hydrophobic or electrostatically charged (Figure 3.2). Two well-studied examples of these approaches are poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (PEG-PPG-PEG, commercially available under the trade name Pluronic) and poly(L-lysine)-graft-poly(ethylene glycol) (PLL-PEG). The former is a triblock copolymer with a central hydrophobic PPG block which preferentially associates to hydrophobic surfaces, while the latter is a graft copolymer with a cationic PLL backbone which adsorbs to anionic surfaces. A range of similar PEG-containing polymer systems have also been reported in the literature, for instance block copolymers with other types of hydrophobic segments and comblike graft copolymers with other cationic backbones, e.g. poly(ethylene imine). Coatings of this type have been shown to produce very low protein adsorption, even from complex biological media such as blood, although they must be designed carefully to achieve a proper packing density of the protein-resistant polymers. The main strength of the physisorption approach is the ease with which the coating can be applied to surfaces, flat as well as curved, often without prior surface conditioning. Incidentally, one of the main weaknesses of the method is the ease with which the coating is again desorbed, since the process is reversible. Adhesion routes based on electrostatics are sensitive to pH and high ionic strengths, while those based on hydrophobic interactions may be displaced if the solvent is changed. One method to increase the strength of adhesion and/or make the coating procedure more universally applicable, is to use “anchor” polymers capable of binding to surfaces through multiple attachment routes.

Recently, a polymer-surface coupling technique which utilizes a biomimetic approach was introduced. This method is based on the highly adhesive properties of L-3,4-dihydroxyphenylalanine (DOPA), an amino acid believed to play a major role in the mussel adhesion system. By coupling DOPA moieties to polymers it is possible to create strongly attached coatings, both on inorganic and organic materials. The exact mechanism of attachment is not completely elucidated; on inorganic materials such as metal oxides physical adsorption is thought to be most important, while the attachment to organic materials seem to follow a different route, involving covalent bonds. In any case, it appears to be a very
promising technique which already has been utilized to fabricate coatings for marine biofouling\textsuperscript{[32]} and biomedical applications.\textsuperscript{[35]}

3.1.2 Chemisorbed coatings

The second group within the “grafting to” approach is based on the use of polymer chains with chemical anchor groups which form covalent bonds with complementary groups on the substrate surface. The polymer chains are typically functionalized with a reactive group in one of the terminal chain ends. A large variety of coupling chemistries may be considered. The chemistry and reactivity of the substrate surface must always be taken into account, and many substrates require pre-modification for successful reactions. Chemically inert plastics generally have to be modified through multistep reactions involving a primary reactivity enhancing step followed by the introduction of the desired chemical groups. The primary step may be achieved by immersing the material in strongly oxidizing solutions, such as aqueous CrO\textsubscript{3}/H\textsubscript{2}SO\textsubscript{4}\textsuperscript{[170]} or by using plasma\textsuperscript{[171]} or other treatments. Such harsh modifications will degrade the surface of the material, and must used with care to avoid damaging the bulk properties.

The routes which allow immediate coupling to untreated substrates through reactions with the appropriate anchor groups are few and highly substrate specific. Silane coupling may be used to bind polymers, as well as smaller molecules, to hydroxyl-exposing surfaces, such as glass and metal oxides (Figure 3.3 B, C). PEG chains with terminal methoxysilane\textsuperscript{[172]} or chlorosilane moieties\textsuperscript{[114, 173]} have been grafted onto glass and other silica substrates using this technique. However, the reproducibility of silane-based techniques is a concern, due to the formation of rather ill-defined monolayers or even multilayers. In addition, the resulting Si-O bonds that hold the polymer in place on the surface are considered to be quite sensitive to hydrolysis.\textsuperscript{[174]}

\[\text{Figure 3.3. Three routes for chemisorption, used in both “grafting to” and “grafting from” methods. A) Thiols on gold. B) Trichlorosilane on silica (e.g. glass). C) Trimethoxysilane on silica. The silanes tend to cross-link with neighboring molecules, which increases the stability of the films.}\]
Self-assembled monolayers of thiols on gold and other noble metals have been used extensively to prepare nonfouling coatings (Figure 3.3 A). The obvious drawback of this technique is the need for very specific substrates, but the unmatched degree of reproducibility and control make them useful as model systems. Most of the experiments performed to elucidate the reasons for the low protein adsorption of PEG have been carried out with the aid of thiol SAMs.

A strength of “grafting to” methods of this type, is that they provide excellent control of the chemistry of the grafted layer, since the grafted chains may be characterized and purified prior to immobilization. With the recent developments in controlled polymerization methods (see below), polymer chains with very well-defined properties and functionalized end-groups can be prepared and grafted to surfaces. On the other hand, the most significant drawback of the “grafting to” approach is the inability to form dense layers of grafted polymer. The kinetics of the adsorption process is slowed down significantly already at partial surface coverage, normally long before all available reactive surface sites are filled. At this point, any candidates for binding to the surface will be sterically hindered by the chains already in place. In addition, a dense polymer layer requires that the polymer chains adopt a stretched conformation, which is not thermodynamically favorable. Consequently, the dry film thicknesses of polymer coatings “grafted to” surfaces rarely exceed 5 nm (the wet thickness may however be significantly higher).

3.2 Coatings prepared by “grafting from” methods

In the “grafting from” reactions described below, the polymer chains are formed in situ, directly from the constituent monomers, on the substrate surface. This has one very important consequence for the properties of the resulting materials. Since the monomers are much smaller than the polymers, the same kinetic diffusion limitations do not apply for the preparation of “grafting from” brushes. Polymers “grafted from” surfaces can consequently form much denser layers than what is possible using the “grafting to” approach.

The literature describing “grafting from” reactions is highly diverse, and the field is constantly evolving. One may differentiate between methods that enable fabrication of controlled polymer architectures and those that do not. The term “grafting from” was coined by Prucker and Rühe as late as 1998, in response to the development towards controlled polymer brush architectures and surface-initiated polymerization. However, methods that qualify under the definition above were described long before that. In the widest sense of the term, the work of Oster and Shibata, who in the late 1950s grafted polyacrylamide onto the surface of natural rubber, may be seen as a starting point for the “grafting from” technique. Most standard polymerization techniques have since been employed for “grafting from” methods. This includes free-radical polymerization and atom transfer radical polymerization (which will be treated in detail below), anionic and cationic polymerization and ring-opening versions thereof, ring-opening metathesis polymerization, Ziegler-Natta
catalyzed polymerization and step-growth polycondensation. Some of these methods are not suitable for the fabrication of nonfouling coatings, while the methods described below all have been used for this purpose to some extent.

3.2.1 Plasma polymerization
Although plasma polymerization in principle is an *in situ* polymerization method, it differs significantly from the other methods treated in this chapter, and is arguably not even a proper "grafting from" reaction, since the initiation of polymerization does not necessarily originate from the surface. The high-energy environment of a plasma (a low-pressure ionized gas) may be used to deposit polymers onto most types of surfaces. The material to be coated is placed in a plasma reactor into which gas-phase monomer is introduced, whereafter the electromagnetic radiation and electron and ion bombardment of the plasma triggers polymerization and surface deposition/grafting. The method enables the use of monomer types which do not contain conventional polymerizable moieties, but ordinary monomers (e.g. methacrylates) can also be used. A typical feature of plasma-polymerization is the formation of relatively thick and highly cross-linked coatings which incorporate fractioned and chemically modified monomer units. The method has been used extensively in biomaterials applications, for instance to prepare coatings composed of cross-linked OEG with excellent protein resistance. An unusual advantage of plasma polymerization is that the method is highly suited for surface modification of samples with complex geometries, and as a consequence it has been shown to be a viable route for rendering the inside surfaces of narrow-diameter tubing blood compatible.

3.2.2 Free-radical polymerization methods
Free-radical polymerization is a classic addition polymerization method which, in its common bulk-, emulsion- or solution polymerization varieties, is applicable to a range of monomer types with substituted double bonds, such as acrylates, methacrylates, vinyl esters, styrenes, and vinyl halides. The reaction is relatively robust and tolerates a wide range of substituted functional groups, requiring only a small addition of free-radical initiator for rapid conversion of the monomer into high molecular weight polymer chains. However, the resulting materials are generally characterized by high polydispersities (large variations in polymer chain length), which may be a drawback in some applications. Several routes have been utilized for the adoption of free-radical polymerization for the fabrication of polymer coatings and brushes, usually adhering to one of four major routes:

1) Free (dissolved) initiator.
2) Covalently bound initiator.
3) Covalently bound monomer.
4) “Initiator-free” methods.
The first route was used by Oster and Shibata, as mentioned above. In this case, a cast film of natural rubber containing the photoinitiator (or photosensitizer) benzophenone was brought in contact with acrylamide monomer and irradiated with ultraviolet (UV) light to initiate polymerization.\[^{179}\] The surface specificity of this particular reaction was perhaps in no small part due to the high local benzophenone concentration at the rubber surface. However, benzophenone also conveys specific chemical surface specificity, as it has an inherent capability to abstract hydrogen atoms from other organic molecules, leading to the formation of free radicals (Figure 3.4 B). If the hydrogen is abstracted from the substrate surface the resulting surface-bound radical may function as a “handle” for initiation of graft polymerization. This technique was significantly refined by Rånby et al.\[^{190-192}\] and was later applied by Rohr et al.\[^{193}\] for the surface modification of a range of untreated plastic substrates with different grafted polymers.

Since the initiator is not bound to the surface in this route, initiation of polymerization in the bulk can never be completely eliminated. However, this may not be a major problem since unattached homopolymer can be removed by thorough rinsing after the reaction is completed. A more serious drawback is the fact that the hydrogen abstraction capability of benzophenone-type initiators is not selective for the substrate, which means that grafted polymer chains are exposed to further hydrogen abstraction, in turn leading to branching and cross-linking of the grafted polymer chains. This technique can thus never be used for the fabrication of true polymer brushes, but is potentially a very quick and convenient method to create covalently bound polymer films on plastic substrates.\[^{191, 194}\]

The most common types of free-radical initiators, such as peroxides and azo compounds, generate free radicals by decomposition when exposed to heat or light. Unlike benzophenone these initiators do not, therefore, form reactive sites on the substrate surface and are hence not suitable for the free initiator route described above. However, decisively surface-specific initiation reactions can be achieved also using this type of initiator, if the initiator molecule is first covalently bound to the substrate surface.\[^{178, 195, 196}\] As the polymerization reaction proceeds from the initiator molecule it – and thus the connection to the surface – remains in
the end of the polymer chain (Figure 3.5). One major advantage of this method, compared with the first route, is that the initiator will not attack the grafted polymer, enabling greater control of the polymer architecture and the formation of true polymer brushes. On the other hand, the need for prior surface functionalization can be challenging in some cases. Materials that permit facile formation of self-assembled monolayers (i.e., gold and silicone oxide surfaces) are therefore amply represented as substrates in the literature. Modified surface-immobilized versions of the common initiator 2,2′-azobisisobutyronitrile (AIBN) have been used extensively these purposes, both with thermal\(^{[197]}\) and photochemical initiation (Figure 3.5 A). The photochemical route allows patternning through photolithographic techniques.\(^{[198]}\) In addition, polymer patterns have been created by first applying the initiator in patterns on the surface through microcontact printing.\(^{[199]}\)

A facile technique to prepare covalently bound initiators has been described by Suzuki et al.,\(^{[200]}\) who exploited the fact that plasma treatment of plastic surfaces lead to the formation of surface-bound peroxide groups. These groups may then be decomposed by UV light or moderate heating, initiating polymerization reactions from the surface (Figure 3.5 B). Similar routes have been developed using ozone pre-treatment.\(^{[201]}\) In addition, Lee et al. used a corona discharge technique with a knife-type electrode for localized modification, enabling the fabrication of density gradients of hydrophilic and nonfouling polymers for biomedical studies.\(^{[202-204]}\)

Figure 3.5. Top: General principle for free-radical polymerization with surface-bound initiators. Bottom: Actual initiator molecules: A) AIBN-type initiator bound to the surface. Note that only one of the radicals remains bound to the surface, although both will initiate polymerization. B) Peroxide groups on the surface may also lead to radical formation.
For the third route, monomer molecules, rather than initiators, are bound to the surface (shown in Figure 3.6). In this case, the polymerization reaction is initiated in solution, and as the macroradicals propagate the immobilized monomer molecules become incorporated in the polymer chains.\[152\] Methacrylate-functionalized silanes have primarily been used as surface-bound monomers.\[205\] It may be noted that this approach has clear similarities with the covalent class of “grafting to” methods, in that a successful reaction of this type demands that the relatively large macroradicals can approach the surface and react with the immobilized monomers. Thus, the same restrictions regarding the maximum achievable polymer chain densities apply. This method has not found particularly widespread use for the preparation of polymer brushes. However, similar methods are routinely applied for increasing the adhesion between inorganic materials and polymers, for instance when preparing glass-supported polyacrylamide gels.\[206\] A major distinction compared with the other methods described in this section is that these gels are polymerized in the presence of cross-linkers, yielding macroscopically thick materials.

**Figure 3.6.** A) Route 3, polymerization with a surface-bound monomer. B) A typical monomer molecule used for these purposes, Methacryloxypropyltrimethoxysilane, here shown bound to a silica surface.

The methods belonging to routes 1-3 all rely on the initiating ability of a dedicated chemical species (the initiator), which is either added to the solution, immobilized, or formed on the substrate surface. However, free-radical polymerization methods which do not require a specific initiator have also been developed. The polymerization reaction still needs to be initiated, of course, but this process is accomplished by other means than the conventional techniques, motivating the terms “initiator-free” and “self-initiated” polymerization. In general, methods classified within this route rely on the supply of relatively large amounts of energy, often in the form of ionizing radiation, which directly excites the monomer molecules and/or chemical groups on the substrate surface, leading to free-radical polymerization. For example, Uchida et al.\[207, 208\] developed a general method to photograft polymer chains onto the surface of untreated plastics without any addition of initiator. In this case the term “initiator free” may perhaps be contested since sodium periodate, which generates radicals when irradiated with UV light, was added in low concentration with the intention to consume dissolved oxygen. This method has later been used to render a range of materials hydrophilic and protein resistant, for instance Poly(dimethyl siloxane) (PDMS).\[209\] The method used for
preparing the hydrogel coatings used in this thesis is a member of the group of initiator-free free-radical techniques, but it will described in its own section below (see Section 3.3).

A related method is radiation grafting,\(^{[210]}\) in which for instance gamma radiation is used, instead of UV light. The method has been used for initiator-free coating of materials such as plastics with nonfouling hydrogel films.\(^{[211]}\) However, it is not in particularly wide use today, most likely due to the practical difficulties associated with the necessary source of radiation, in combination with the risk of radiation damage to the substrate and the relatively low control of thickness and other surface properties afforded by the process.\(^{[187]}\)

### 3.2.3 Surface-initiated controlled polymerization methods

During the last decade, the research focus in the field of polymer brush preparation has largely been shifted towards the recently developed living/controlled polymerization methods. While the preparatory requirements (surface pre-modification etc.) are similar to those needed for free-radical polymerization, the new methods allow greatly enhanced control of the composition, molecular weight (MW) and architecture of the synthesized polymer. The methods have been applied for preparing polymer brushes with novel and interesting properties.\(^{[4]}\) Although none of these techniques have been used in this work, an introduction to the most commonly used methods, as well as some applications within relevant fields, will be presented here to provide a context for the work. For a comprehensive review of surface-initiated controlled polymerization techniques and what has been accomplished through them to date, see e.g. the recent review by Barbey et al.\(^{[212]}\)

**Surface-initiated atom transfer radical polymerization (SI-ATRP)** is the most commonly used polymerization method for controlled “grafting from” reactions.\(^{[212]}\) The original descriptions of solution phase ATRP were published in 1995\(^{[213, 214]}\) and the first example of polymer brushes grafted from solid surfaces with SI-ATRP was reported a few years later.\(^{[215]}\) The method is still developing, but has already been used to prepare hundreds of types of polymer brushes, often intended for biomedical or biofouling applications.\(^{[212]}\) The greatest strength of the method, compared with normal free-radical polymerization, is that the termination of the polymerization reaction can be controlled precisely, enabling the synthesis of polymers with very even MW distributions or of block copolymers. This is possible since ATRP permits that a majority of the polymer chains are “living” (with potential to grow), although dormant, at any time. A catalyst system consisting of a transition metal, often copper, and a nitrogen-containing complexing ligand interact with the propagating macroradical, transferring a halogen atom (generally bromine or chlorine) to it, stopping the propagation of the polymerization reaction (Figure 3.7). Since this deactivation stage is a reversible redox reaction, the dormant species may later become re-activated, continuing the addition of monomer molecules to the polymer chain end, until it reacts with the transition metal complex once more. The actual polymerization reaction proceeds like in normal free-radical polymerization, but instead of propagating until termination (i.e. death) occurs, only a few monomer molecules are added to the macroradical in each activation cycle, since the chemical
equilibrium strongly favors the dormant state. The concentration of propagating chains is thus always very low in the system, which limits the risk of termination by radical coupling. Hence, the polymer chains can “live” for hours rather than seconds, which is the case for conventional free-radical polymerization.\cite{216, 217} This long lifetime and the uniform growth of the polymer chains make it possible to manipulate the polymerization conditions during the reaction, for instance by adding additional monomer species to form block copolymers. The versatility of SI-ATRP also enables the construction of many other complex polymer architectures which were previously near impossible to achieve, such as surface-bound polymer gradients with changing composition or MW.\cite{218}

Figure 3.7. A) General reaction route for ATRP. The transition metal-ligand complex (Cu-L) initiates the reaction by transferring the X atom (a halogen atom) from the initiator molecule, forming a free radical. The ensuing polymerization reaction continues until an X atom is transferred back to the macroradical. B) Typical bromine-containing surface-bound initiator (here shown bound to a gold surface) and a Cu - 2,2'-bipyridine complex. Additional free initiator is often added to the monomer solution to ensure good reaction control. Adapted from Braunecker et al.\cite{216}

Initiation in ATRP processes takes place in the same way as the activation reaction described above, with the transition metal catalyst removing a halogen atom from the initiator molecule, thus creating a radical from which polymerization then originates. If the initiator is immobilized to a surface, the polymer chain will retain this link. Like most other “grafting from” reactions, this demands reproducible and well-controlled techniques for initiator immobilization, and SI-ATRP has hence mainly been applied to substrates that permit coupling with silane or thiol chemistries. For inert polymer substrates, the situation is similar to that of the covalent “grafting to” methods described above; some type of preparatory step, such as chemical oxidation or plasma treatment is generally necessary. It may also be noted that surfaces suitable for free-radical “grafting from” reactions can be used also for SI-ATRP reactions (in a related reaction referred to as reverse SI-ATRP), which opens up further possibilities, for instance the direct use of plasma-generated peroxides for the initiation process. A recent work by Jin et al., who prepared poly(OEG methacrylate) brushes on plasma-treated polyurethane for increased protein resistance,\cite{219} is a step in this direction.
Physisorbed initiator-functionalized cationic macromolecules have also been used to graft polymer brushes from negatively charged surfaces.\textsuperscript{[220]}

SI-ATRP has been used extensively to prepare nonfouling polymer brushes of different types, both based on PEGs and zwitterionic groups. Like free-radical polymerization reactions, the most commonly used monomers for this purpose are (meth)acrylates or methacrylamides with side chains incorporating either zwitterionic\textsuperscript{[34, 122, 221, 222]} moieties or relatively short PEGs (~5-25 units) with hydroxyl or methoxy ends groups.\textsuperscript{[113, 115, 223, 224]} Differently charged monomers have also been copolymerized to achieve protein resistant charge-balanced coatings.\textsuperscript{[129, 130]} A few other monomers, such as 2-hydroxyethyl methacrylate, have also been used with some success in similar applications.\textsuperscript{[225, 226]}

One issue that needs to be considered regarding SI-ATRP is the cytotoxicity of the commonly used copper catalyst. This is a potential problem for biomedical applications, and may in addition lead to complications when developing decisively non-biocidal coatings for marine biofouling applications. Methods to decrease the copper content\textsuperscript{[227]} or use other less toxic catalysts have therefore been developed. In addition, ATRP does not tolerate as many monomers as normal free-radical polymerization does; for instance, carboxylic acid-containing monomers tend to poison the ATRP catalyst by coordinating to the transition metal.\textsuperscript{[217]} Methods to fabricate carboxylic acid-containing brushes are however available, either by the use of monomers with temporarily protected acids,\textsuperscript{[228]} post-functionalization\textsuperscript{[220]} or modified synthesis protocols.\textsuperscript{[229]}

Nitroxide-mediated polymerization (NMP) has similar characteristics to ATRP, in that the propagating macroradical can be reversibly converted into a dormant state, which enables high control of the polymer chain length. This is accomplished by the use of alkoxyamine-containing initiators, which produce one normal free radical and one stable radical species upon homolytic cleavage.\textsuperscript{[230]} Surface-initiated NMP (SI-NMP) is made possible by immobilizing the initiator to the surface.\textsuperscript{[231]} Compared with ATRP, the reaction has the advantage that no metal catalyst is required. However, the method is not particularly flexible in terms of monomer choice, since styrene-type monomers are typically required for good results. In addition, the reactions demand higher temperatures than ATRP for activation.\textsuperscript{[212]} In a few instances, SI-NMP has been used to prepare nonfouling polymer brushes. An example is the PEG-containing coatings prepared by Andruzzi et al.\textsuperscript{[232]} as bioselective surfaces. Special PEG-substituted styrene monomers had to be synthesized for this purpose, since the corresponding PEG-containing methacrylates (which are commercially available) are not compatible with SI-NMP.

Reversible addition-fragmentation chain transfer polymerization (RAFT) is yet another controlled polymerization method developed\textsuperscript{[233]} and adopted for surface-initiated polymer brush formation\textsuperscript{[234, 235]} in recent years. Specific chain transfer agents (thiocarbonylthio compounds, most often dithioesters) are used in RAFT for transient “capping” of the propagating macroradicals. The polymer-bound transfer agent may then react with another
propagating chain end, leaving an active radical in its place. Propagation ensues until an additional transfer agent molecule is encountered, and so on.\textsuperscript{236, 237} The equilibrium between dormant species and active radicals needs to allow the concentration of propagating radicals to be kept low at any time, to minimize coupling reactions between propagating macroradicals. Normal initiators can be used for the primary generation of radicals; in fact, free-radical polymerization reactions can, at least in principle, easily be converted into RAFT reactions by simply adding a RAFT transfer agent. In addition, the reaction is compatible with almost all monomers accessible for free-radical polymerization. For surface initiated RAFT, there are two main ways to achieve polymer brushes bound to the surface: either the surface is functionalized with initiator or with RAFT agent. The latter case may lead either to a “grafting to” situation or a more strict “grafting from” reaction, depending on how the RAFT agent is attached to the surface.\textsuperscript{238} Surface-bound initiator leads to a “grafting from” reaction.\textsuperscript{234}

SI-RAFT has been used to prepare grafted films of hydrophilic polymers on different substrates, although not as frequently as SI-ATRP. A notable example is the work of Yoshikawa et al., who used a plasma treated a fluoropolymer as a peroxide-exposing substrate from which poly(2-hydroxyethyl methacrylate) could be grafted.\textsuperscript{239} Grafting of PEG-containing polymers via the conventional free-radical route, i.e. by first immobilizing an AIBN-type initiator after chemical oxidation of the surface, has also been demonstrated for a similar substrate.\textsuperscript{240}

\textbf{Photoiniferter}s (PIs) are photoinitiators which may also act as chain transfer agents and terminating species in a polymerization reaction (\textit{initiation, transfer, termination}).\textsuperscript{241} The “termination” intended here is in fact the recombination of the relatively stable iniferter radical with the propagating macroradical. This state is not necessarily permanent, however; the compound may undergo photolysis to reform the macroradical, provided that the system is irradiated with UV light. Surface-initiated PI-mediated polymerization can be accomplished by immobilizing the PI to the surface.\textsuperscript{242, 243} Compared with the methods described above, SI-PI-mediated reactions are not quite as well-controlled,\textsuperscript{244} but the method still has a significant value in its inherent capability for photopatterning.\textsuperscript{245} This technique has been used to prepare patterned nonfouling/cell-binding coatings and for the construction of integrated microfluidic devices.\textsuperscript{246}

\section*{3.3 The SIPGP method}

\subsection*{3.3.1 Practical aspects}

The coating technique developed in this work is a “grafting from” reaction, belonging to the group of UV-initiated free-radical polymerization reactions. It will be referred to as SIPGP (self-initiated photografting and photopolymerization) in this chapter, for simplicity. It is a
practically very simple method, involving only the following components: 1) a substrate to be coated, 2) a monomer solution, 3) a UV light source and, finally, 4) a UV-transparent rigid plate. The practical requirements for each of these components will be discussed below.

The substrate needs to be organic in nature, or at least be covered with an organic film. We have tested a range of polymers as substrates and most of them may be used without pre-modification. For instance, polystyrene, polyethylene, poly(methyl methacrylate), and cyclic copolymers (Zeonor) may be used as substrates. PDMS appears to need prior plasma treatment, although we have not investigated that process thoroughly. In addition, different SAMs may be used. A methacrylate-terminated silane SAM (shown in Figure 3.6 B) was used in Paper II, but the functionality exposed to the monomer solution actually appears to be relatively unimportant. Metals, metal oxides and silica surfaces cannot be grafted without prior modification, for instance by first coating them with an organic SAM. The substrates need to be flat for optimum results, although minor deviations may be acceptable. However, curved and irregular samples cannot be evenly coated using this method.

The monomer solution only contains monomer and a solvent. We have found that high concentrations of monomer do not generally lead to good results, and therefore the concentration is usually kept between 1-10%. Methacrylate monomers are preferred (for a general chemical structure see Figure 2.7 E), although most classes of monomers susceptible to free-radical polymerization appear to be possible to use. The solvent for the monomer solution has been water in most cases, mainly since the monomers we have been interested in happen to be water-soluble. For monomers with relatively low solubility in water, we have used ethanol-water mixtures. Organic solvents have rarely been used, and would in any case be difficult to handle due to the risk of dissolution of the substrate polymer (unless a SAM-based approach is used). We have not purified the monomers after receiving them from the suppliers and we have not removed dissolved oxygen from the monomer solutions.

Several types of light sources have been used, although almost all the experiments described in this thesis have been performed using only one of them; a tubular Philips TUV PL-L 18W UV-C low-pressure mercury lamp. The power of this lamp is very low compared with most other UV lamps used for polymerization. As a side note, it may be noted that the output of the lamp seems to be relatively similar to that of the lamp used by Oster and Shibata in 1957, which was described as a “15 W germicidal lamp”. It is a very convenient lamp type since it does not produce much heat, and in addition it irradiates a large area, allowing the use of relatively large samples (such as the glass slides used in Paper II). We have also utilized the large irradiated area for the fabrication of gradients. The other main light source used is a Newport Apex 100 W high-pressure mercury lamp. It produces a focused and collimated beam of light which is ideal for photolithography. The irradiated area is however not larger than a few cm². The light from this lamp has usually been filtered with a dichroic mirror to only provide wavelengths between 240-255 nm. In any of these cases, it is necessary to contain the UV light since it is potentially quite harmful. For the TUV lamp, a reactor, which
basically amounts to a steel box with the lamp inside, has been built. The atmosphere inside the box can be controlled by purging with e.g. nitrogen.

We have almost exclusively used quartz discs as the necessary UV-transparent rigid plate in our experiments. However, several other options may be considered as well. In the initial experiments, we used polyethylene film. We have also used PDMS and the cycloolefin Zeonor. All these materials transmit UV-C light sufficiently well for the reaction to take place. Quartz is convenient since it is flat and rigid, and can be washed and reused. In addition, it is a suitable material for photomask fabrication.

The method itself is described in the papers in this thesis, in particular in Paper I. The monomer solution is mixed, and the quartz plate is cleaned in ammonia/hydrogen peroxide at 85 °C and then thoroughly washed with water. The substrate is prepared (cut, rinsed, etc.) and placed close to the reactor. It is best if the substrate is placed on a pedestal-type base or at least on a hydrophobic material, such as an overturned polystyrene Petri dish. The monomer solution, typically 5-7 µL per cm² of sample area, is then placed on the sample with a micropipette. The quartz plate is thereafter lowered towards the sample and brought in contact with the drop, so that the monomer solution spreads between the two surfaces. The “sandwich” assembly, with the substrate hanging underneath the quartz plate by capillary forces, is placed in the reactor, under the lamp, and is irradiated for 1-10 minutes (the setup is schematically shown in Figure 3.8). Afterwards the “sandwich” is washed with water/ethanol until the sample is released. Ultrasonication is sometimes necessary for removing the sample. Further rinsing of the sample completes the process.

Some variations of the method above have also been developed. The thickness of the grafted film depends on the irradiation time. Therefore, it is possible to create gradients by pulling a shutter across the substrate during the irradiation phase.\[78\] The result is a linearly varying

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**Figure 3.8.** Cartoon of the reactor setup for the SIPGP method. The lamp and sample is contained in a steel box during irradiation. The magnified view shows a propagating macroradical (center) and a recently photoexcited monomer, ready to abstract a hydrogen from the surface and create a new site from which grafting can be initiated.
irradiation dose, which normally leads to a thickness gradient in the coated film. This method was used in Paper III to fabricate electrostatic charge gradients. Photolithographic patterning is also easily accessible. A photomask, conveniently integrated on the quartz plate in form of a metal pattern, has been used for the patterning processes. As noted above, a collimated light source is superior to the TUV lamp in patterning applications, although larger patterns (~100 µm) can be fabricated in the TUV reactor. Paper V shows examples of patterning, including patterned gradients.

3.3.2 The SIPGP mechanism

The exact chemical mechanism behind the technique described above is not completely resolved, and it should be said that the work carried out in Linköping has mainly focused on the applications, rather than the chemistry, of the method. It is obvious that the initiation of the reaction takes place through radical formation, due to the fragmentation of molecules, caused by the highly energetic UV light. Since the method can be used to coat substrates which do not absorb UV light, photoexcitation of monomer molecules appears to be the primary effect responsible for the initiation reaction. Deng et al. suggested that a fraction of the monomer molecules would become excited by the UV light and subsequently abstract a hydrogen atom from the surface, creating a surface-bound radical. The polymerization could then proceed from this point, forming a polymer chain “grafted from” the substrate. A tentative reaction scheme is shown below (Scheme 3.1). According to this explanation, the success of the grafting reaction should depend significantly on the bond dissociation energy of the hydrogen atoms available on the surface. This hypothesis has recently been empirically
verified by Steenackers et al.\textsuperscript{[248, 249]} who studied the differences in grafting density on different materials and found that hydrogen bond dissociation energies below approximately 100 kcal/mol (~420 kJ/mol) are necessary for the hydrogen abstraction reaction to take place. This would mean that most hydrocarbons\textsuperscript{[250]} are possible to coat using this method, while some other hydrogen-exposing surface chemistries are inert.

It is necessary to observe that several other photochemically driven processes may take place in parallel with the grafting/polymerization reaction, including polymer degradation. The sensitivity to photodegradation differs between polymers and the resulting effects commonly include oxidation, chain scission and/or cross-linking.\textsuperscript{[2]} These effects were studied in Paper I and must be taken into account when setting up the process and investigating the results. Unfortunately, these effects are difficult to avoid altogether.

3.3.3 SIPGP in comparison with other methods

Photoinitiated grafting for modification of polymer surfaces has, as discussed above, been in use for several decades. In the initial development work for the hydrogel coatings described in this thesis, the work of Rånby et al., as mentioned above, was a significant source of inspiration. The initially intended application for the hydrogel coatings (as sensor surfaces for disposable bioanalytical devices) demanded that the hydrogel films could be coated directly onto plastic surfaces, preferably in a single-step reaction. This requirement meant that many of the aforementioned polymerization techniques, however elegant and versatile, were deemed to be too complex for implementation. However, the work of Rånby indicated that one-step fabrications of coatings could be feasible through a free-radical reaction initiated by UV-excited benzophenone. Consequently, benzophenone was added to the monomer solutions in the initial work performed in our lab. In our hands, the preliminary experiments were successful in terms of initiation of polymerization but appeared to produce thick and heavily cross-linked films. In an attempt to optimize the benzophenone concentration, monomer solutions with different benzophenone concentrations were tested, including a “negative reference” with no addition of photosensitizer. Surprisingly to us, we found that also this reaction led to grafted polymer films. At this point, we surveyed the literature and found a few references to similar results. Interestingly, it was again Rånby and co-workers who were first to describe this effect in a conclusive manner.\textsuperscript{[247]} It is intriguing to note that the routes to discovery appear to have been similar in many cases, with an initial experimental plan using photosensitizers/initiators and subsequent realization that the exclusion of all additives (except the monomers) still produced acceptable results. We also focused our attention to the experiments described by Suzuki et al.,\textsuperscript{[200]} and Jeong and Lee,\textsuperscript{[204]} (discussed above) who showed that pre-treated materials with immobilized peroxide moieties could initiate grafting when energy in the form of heat or light was supplied. Since we used plasma treated substrates at this point, this mechanism appeared to be a viable explanation for the results. However, as further experiments showed that also non-plasma treated plastic substrates, and indeed most organic materials, were possible to graft without the addition of
photosensitizer, the mechanism again appeared to us to be mainly photochemically driven, as
described by Rånby\cite{247} and further studied by Wang and Brown a few years later.\cite{251}

More recently, Steenackers et al. developed a similar method which makes use of long-
wavelength UV light (>300 nm) for free-radical photopolymerization reactions without any
added initiator.\cite{248, 249, 252-254} They began referring to this method as SIPGP (self-initiated
photografting and photopolymerization), a term which was lately also adopted also by us, and
is used here and in some of the papers in this thesis. The term actually appears to originate
from Wang and Brown, who titled their 2004 paper\cite{251} “Self-initiated photopolymerization
and photografting of acrylic monomers”, although they never used the actual acronym.
However, there are some significant differences between the method used in that paper and
later by us, and the SIPGP method, as developed by Steenackers. Wang and Brown (building
on the work done previously by Rånby) used an unfiltered medium-pressure UV lamp which
gave a light intensity of 24 mW/cm² in the 250-260 nm region. They irradiated their monomer
solutions through a thin film of high-density polyethylene, which transmits light relatively
well at these wavelengths. The irradiation time never exceeded 1 minute in the experiments
described, and this yielded grafted polymer from a range of monomers (2-hydroxyethyl
(meth)acrylate, (meth)acrylic acid, glycidyl acrylate and PEG methacrylate). In the method
used by Steenackers the samples are kept in a glass vial while irradiated. This effectively
filters the light so that only relatively long UV wavelengths reach the sample. In addition, the
UV source has a maximum intensity at 350 nm. The use of only long wavelength UV light is
intentional, and is meant to protect the grafted polymer from photodegradation.\cite{255} However,
this modification of the protocol also leads to much longer reaction times, typically 16 hours.

\textbf{Figure 3.9} illustrates the difference between using unfiltered light and light filtered to
resemble the conditions used by Steenackers et al. Clearly, the kinetics, and possibly also the
mechanism, of the grafting reaction are different in these respective cases. The method
developed by us has more in common with that of Wang and Brown, since we use a mercury
lamp with a main emission peak at 254 nm and a fully UV transparent quartz plate between
the lamp and the monomer solution. The irradiation time is also relatively short (no more than
10 min), which implies that the mechanism is similar.

Compared with the controlled polymerization methods described previously in this chapter,
the SIPGP method provides much less control of the resulting polymer architecture. It is
difficult to estimate the polymer MW since the polymerization reaction cannot be expected to
proceed under the same conditions at the surface as it does in solution. Once the polymer is
attached, it is not easily removed, which makes it difficult to measure the MW. However, a
broad distribution in MW is expected, as in all free-radical reactions. One significant feature
of the SIPGP method is its low surface selectivity in terms of the substrate. The advantages of
this are obvious, as it enables extremely facile surface modification and coating of many
important materials. The negative aspect is that it will also inevitably lead to a highly
branched chain structure, as new grafts grow from previously grafted chains. Hence, the
polymer architecture should not be described as a brush, in the conventional sense, but
perhaps more suitably as a “bush”. The same effect may lead to cross-linking, for instance by combination of macroradicals. This effect also has implications for the properties of the resulting coatings, which appear to be very dense, as shown by their very high resistance to diffusion of proteins.\cite{78}

![Figure 3.9](image)

**Figure 3.9.** Infrared spectra measured in two different locations on a sample, which was partly covered with a glass coverslip during irradiation with a high-pressure mercury lamp for 6 minutes. The coverslip absorbed light with wavelengths below 300 nm, as shown in the inset. The solid spectrum was measured in an area exposed to filtered light, while the dotted spectrum was measured in an area exposed to unfiltered light. As is evident from the large differences in absorption intensity, light with wavelengths < 300 nm is necessary for the grafting reaction to take place under these circumstances.

**In conclusion,** the following main strengths and weaknesses of our approach to SIPGP may be distinguished:

**Strengths:**
The method is quick and simple, and requires few preparations. The necessary equipment is not expensive, and no exotic chemicals are needed. A large array of monomers may be used, and the consumption of monomer per coated area is very low. A wide range of substrates can be coated without any prior modification. The coatings are generally very stable and can potentially provide attractive interfacial properties, depending on the application and the choice of monomers. Gradients and patterns can be fabricated relatively easily.

**Weaknesses:**
The formed polymers are poorly defined in terms of the polymer architecture, but they are certainly branched and dense, which is a drawback in applications such as affinity sensing, for which they were originally intended. The coatings are also difficult to characterize in terms of MW and degree of cross-linking. The reproducibility can be poor for some monomer systems, and relatively rigorous optimization is needed for each new monomer system. Degradation and other undesired processes may occur in parallel with polymerization. The method does not (in our setup) work for non-flat samples.
The synthesis of ultrathin coatings would be futile without sensitive characterization techniques. The data interpretation is often greatly facilitated if several complementing methods are used in combination. The three techniques discussed below were chosen from a larger body of methods used during this work. Atomic force microscopy, null-ellipsometry, spectroscopic ellipsometry, scanning electron microscopy, fluorescence microscopy, x-ray photoelectron spectroscopy, contact angle goniometry, quartz crystal microbalance, impedance spectroscopy and many other techniques have also been used to characterize the hydrogel coatings fabricated in our laboratory.

4.1 Infrared spectroscopy

Infrared spectroscopy (IR) is used to measure vibrational energy transitions, yielding information about the types of covalent bonds present within a material. Measurements are carried out by irradiating the sample with infrared light (commonly with wavelengths of about 2.5 – 25 micrometers, or 4000 to 400 cm\(^{-1}\), for the “mid-IR” region\(^{[256]}\)) and determining the wavelengths at which absorption takes place. Only those molecular vibrations that lead to a change in the dipole moment upon vibrational excitation can absorb energy from the IR light. The energies of the molecular vibrations (and hence of the absorbed light) can be estimated from the following expression:\(^{[257]}\)

\[
\nu = \frac{1}{2\pi c} \sqrt{\frac{k}{m^*}}
\]

where \(k\) is the force constant and \(m^*\) is the reduced mass \((m_1m_2/(m_1+m_2))\) of the two vibrating atoms. This formula, which is valid for small molecular displacements, is in fact the same as that used for harmonic vibrations in classical mechanics, with \(k\) as the spring constant.\(^{[257]}\) A high force constant and low reduced mass will consequently lead to a high vibrational frequency (and a high wavenumber, \(\nu\), which is the conventional quantity used in IR spectroscopy). In addition, quantum theory states that any allowed energies must fulfill

\[
E = \hbar \nu \left[ n + \frac{1}{2} \right]
\]

with \(n = 0, 1, 2 \ldots\)

for this somewhat simplified model with equidistant energy levels.\(^{[257]}\) The absorption of light is thus quantized, with very narrow absorption bands for each type of bond. Since the exact
vibrational energy is highly dependent on the type of bond, the atoms involved, and the local chemical environment, interpretation of absorption spectra can yield a wealth of data relating to the chemistry of the sample. The measurement procedure is relatively rapid, since the Fourier transform IR spectrometers used today allow a single measurement to be performed in seconds. Usually, hundreds of successive measurements are added together to increase the signal-to-noise ratio of the final spectrum.

Infrared spectroscopy is one of the most widely used methods for chemical characterization of polymers. In addition, the technique is highly suited for analysis of ultrathin films prepared on metal surfaces. Together, these factors have made it an indispensable routine characterization method for the grafted hydrogels described in this thesis. IR spectroscopy offers significant flexibility in terms of sampling methods and sample types, and allows in situ measurements of surface-bound films, although “wet” measurements are unfortunately rather complicated. The measurement mode is selected depending on what is suitable for the sample in question. The most common sampling methods include transmission (semi-thin films, and general analysis of chemical compounds), external reflection (thin films on reflecting substrates), reflection-absorption (extremely thin films on metal substrates), internal reflection (thick, soft materials and thin films prepared on such, and “wet” measurements), and diffuse reflection (powders and poorly reflecting materials). All these sampling modes, except diffuse reflection, have been used at different stages in the development process of the coatings described herein. In addition, each sampling method may be adapted to different instrumental configurations, typically either macro-scale measurements or microscopy.

IR microscopy was used for the characterization of the gradient surfaces in Paper III (Figure 4.1). This is potentially a very powerful technique in cases when the chemical composition of a sample needs to be determined in a single point, or along a line. The high lateral resolution is most often achieved by focusing the IR light on a small point on the surface. For the highest possible resolution, a focal plane array (FPA) multiple element detector, capable of measuring one unique spectrum per detector element, may be used. Such a device makes it possible to map out the chemical features of an area in a single measurement, with a resolution approaching the diffraction limit of the infrared light.

### 4.2 Surface plasmon resonance

Instruments utilizing the surface plasmon resonance (SPR) effect are routinely used for analyzing and quantifying biomolecular interactions. The method is based on the optical excitation of surface plasmons in a thin metal film. Gold is normally used as the metal. The surface plasmon will only be excited by incident light under certain conditions, in particular with specific wavelengths and angles of incidence. If the local environment next to the surface changes (in terms of the dielectric constant, or refractive index) the conditions for excitation will shift. This shift is therefore a function of the local refractive index, and may be related to
events taking place next to the metal surface, for example an increased protein concentration due to receptor-ligand binding. Fractions of adsorbed protein monolayers can be detected using this technique, which is obviously useful when testing the properties of nonfouling coatings. The maximum probe depth is on the order of a few hundred nm,[261] and events taking place further away from the surface will consequently not be detected.

The commercially available instruments typically provide data in the form of integrated signals from a few discrete areas on the sensor surface. However, SPR can also be used as an imaging technique, by illuminating the sensor surface evenly and collecting the reflected image with an array detector.[262] This technique is here referred to as imaging SPR (iSPR). It is a very versatile and useful technique, as is indicated by the diverse applications of iSPR demonstrated in this work. In Paper III, iSPR was used to study protein adsorption along a gradient (Figure 4.1), in Paper V the technique was used to study cell adhesion, and in Papers VII and VIII it was used to investigate the behavior of barnacle cyprids. Much of the versatility and attraction of the method lies in the extreme surface sensitivity and the fact that analytes do not need to be labeled to be detectable.

A custom-built iSPR system,[261] designed, constructed and primarily handled by Olof Andersson, has been used in this work. In the normal measurement mode, the wavelength of the incident light is scanned between ~600-800 nm at a constant angle of incidence. One such scan takes about one minute to perform. The instrument also allows qualitative real-time measurements, if both the angle of incidence and the wavelength are fixed while the intensity changes in the reflected light are observed. This was the method used for tracking the movements of barnacle cyprids in Papers VII and VIII. The focused field of view of the instrument is 2.0 x 0.5 mm², with a lateral resolution of ≈10 µm.[261] Since the incident light impinges upon the sample from below, the space above the sample surface is accessible for the connection of auxiliary components, for instance a microfluidic flow system. This option was used for the gradient studies in Paper III. It may be noted that the high surface sensitivity makes it difficult to use the method to its full potential for thicker films and that special gold-coated glass substrates are required.
4.3 Neutron reflectometry

Neutron reflectometry is by far the most exotic of the methods used in this thesis, due to the extremely costly and advanced research infrastructure needed to conduct the experiments. The experiments described in Paper VI were carried out at the Institut Laue-Langevin in Grenoble, France, which is an international research facility for neutron-based experimental techniques. A research reactor at the site provides one of the most intense neutron sources in the world. The neutrons are led to different instruments placed outside the reactor enclosure. Some of these instruments are neutron reflectometers, of which we have used two; ADAM and D17.

Neutrons are not charged, and interact with other atoms only by nuclear-nuclear interactions, which are extremely short-range in nature. Like light, neutrons exhibit particle-wave duality and may be reflected or transmitted with a change in direction (refracted) when reaching an interface where the refractive index is altered. Neutron reflectometry therefore has great similarities with the related method x-ray reflectometry. However, the neutron refractive index, $n$, for a material is totally different from that of photons, and depends on the properties
of the atomic nucleus rather than the surrounding electrons.\textsuperscript{[263]} It is therefore very isotope dependent. The refractive index can be approximated with the formula\textsuperscript{[264]}

\[ n \approx 1 - \frac{\lambda^2 \rho}{2\pi} \]

where $\lambda$ is the de Broglie wavelength of the neutrons and $\rho$ is the scattering length density. Since most materials have positive $\rho$, the refractive index is usually lower than in vacuum. $\rho$ depends on the average number of atomic nuclei per volume and the scattering length of these nuclei. The scattering length, in turn, is a measure of the strength of the interaction between the atomic nucleus and neutrons.\textsuperscript{[264]} $\rho$ is a characteristic feature of a material and can be found in standard tables.

In a neutron reflectometry measurement, neutrons are directed towards the surface of a material at a grazing angle and the specularly reflected intensity is measured. The intensity will drop off with increasing angles as a greater portion of the neutrons will be transmitted into the material. The proportion of transmitted/reflected neutrons will obey the same Fresnel conditions as those applying for s-polarized light, and can therefore be treated with standard optics, taking into account the angles involved and refractive indices of the materials.\textsuperscript{[263]} However, if there is a thin film on the surface, the neutrons may be reflected also at the second interface, leading to interference in the reflected neutron beam. If there are more films than one, the appearance of the reflected intensity curve will be even more complex. The interpretation of the reflectivity becomes more difficult in these cases, but also potentially more rewarding, since the interference pattern as the angle is changed will contain information about the thin film(s) on the surface, or more generally, the compositional inhomogeneities normal to the interface. According to the so-called kinematic approximation, it is possible to fit a density profile to the reflectivity curve using the following approximation\textsuperscript{[265]}

\[ R(q) = \frac{16\pi^2}{q^2} |\hat{\rho}(q)|^2 \]

Where $R(q)$ is the reflected light as a function of the so-called wave vector transfer $q$ ($q = 4\pi \sin \theta/\lambda$), which is consequently used as the x-axis variable when plotting neutron reflectivity data. $\hat{\rho}(q)$ is here the Fourier transform of the scattering length density profile. The data is interpreted by fitting a model containing a number of layers with different scattering length densities to the experimental data, and analyzing how well they agree.\textsuperscript{[263]} If the surface is rough or there is interpenetration between layers the layer model needs to take also this into account, since it will affect the reflectivity significantly. Many different layer profiles may give rise to the same reflectivity curve, and therefore it is difficult to be certain about the validity of the fit for a single measurement. To resolve this, it is possible to vary the contrast within the layers by changing the isotope composition, normally by deuteration. Provided that all other properties of the deuterated/non-deuterated films are the same, a few of these measurements can together provide a dataset that can be interpreted without ambiguity. In addition, it is possible to use the special properties of water, which in its H\textsubscript{2}O form has a
negative $\rho$ while the $\rho$ of D$_2$O is positive. By mixing these liquids any intermediate $\rho$ can be achieved, which makes it possible to exactly match most other materials, for example the silicon substrates used in Paper VI.

Neutron reflectometry has some unique features which motivate its use despite the great practical challenges involved.\cite{264} It is unusually sensitive to light atoms, which makes it ideal for analyzing systems composed of organic molecules, such as polymer coatings. The thickness and the composition of such systems can be determined simultaneously with neutron reflectometry. The resolution of the method is very good since the wavelength of the neutrons is typically in the order of Ångströms. In addition, few materials absorb neutrons strongly which makes it possible for the neutron beam to reach interfaces buried deep inside materials, or inside sample chambers holding liquid or controlled atmospheres.\cite{264} It is therefore an ideal technique to study e.g. adsorption or swelling phenomena, as in Paper VI. In addition, neutron reflectometry is a non-destructive method which ensures that degradation of the sample does not occur during the measurement process. Neutron reflectometry is also sensitive to the magnetic properties of the material,\cite{264} although that aspect has not been studied in this work.
Photographed poly(ethylene glycol) matrix for affinity interaction studies.

Larsson A, Ekblad T, Andersson O and Liedberg B.

Biomacromolecules, 2007, (8), 1, 287-295.

Contribution: Contributed significantly to the development of the photografting method, performed the non-specific protein adsorption experiments and wrote a section of the manuscript.

Description and comments: This was the first of a series of papers from our lab which focused on photografted hydrogels. The intended use for these materials was as matrices in immunoassay-type applications, particularly for biosensors. Copolymers of 2-hydroxyethyl methacrylate (HEMA) and PEG methacrylate (PEGMA) were grafted as 100-700 Å coatings on spin-coated Zeonor. We originally developed the method for use on plastic substrates, but in this paper spin-coated analogues were used to enable sensitive studies with ellipsometry and IR spectroscopy. The substrates were plasma treated before they were coated with hydrogel. Some aspects of the development work is described in chapter 3 in this thesis. The grafting kinetics (thickness as a function of irradiation time) and the degrading effects of the UV light are discussed in the paper. It is evident that the two comonomers are differently affected by the UV light – the HEMA component appears to be much more sensitive than PEGMA. The copolymer composition as a function of the monomer feed ratio was studied with IR spectroscopy. The eventual choice of the 1:1 molar ratio of the two monomers was not arbitrary – we had empirically found that a concentration ratio in this range gave the most reproducible results. This version of the hydrogel was also very protein resistant, as indicated by the low adsorption from fibrinogen solution and blood serum/plasma. We have later noticed that also PHEMA hydrogels fabricated using this method are very resistant to adsorption of most proteins (see Paper IV). This had not been investigated at the time of writing, however, which explains the discussion about the likely poor protein resistance of PHEMA. The post-modification of the hydrogel was performed with bromoacetic acid/NaOH. After this treatment, protein could be immobilized to the material, as shown with infrared spectroscopy and SPR. An affinity interaction study was performed using a model protein system.
II Poly(ethylene glycol)-containing hydrogel surfaces for antifouling applications in marine and freshwater environments.


Contribution: Planned the work, fabricated the samples together with G. Bergström. Performed several of the bioevaluation experiments together with the responsible partners at the test sites. Wrote the manuscript.

Description and comments: In the AMBIO project, we originally wanted to use the newly developed hydrogels to fabricate surfaces with different chemical functionalities, bound to the hydrogel in patterns. Before we could do that we had to know more about the biofouling properties of the hydrogel itself. Obviously, we knew that it was protein resistant so we also thought it would be interesting to see how this might influence the results of the biofouling assays. The assays required glass slides, and therefore we developed the silanization procedure, using a methacrylate-functionalized silane. Hanna Lassus, who was a Master’s student supervised by me at the time, helped developing the silanization protocol. Although relatively time consuming due to the large sample batches involved, the coating procedure appeared to work well but our main problem was that glass slides are so difficult to use as substrates for characterization. We resorted to silicon and gold for ellipsometry and IR, respectively. The actual coatings sent for biofouling assays were “quality controlled” by measuring their advancing and receding contact angles.

I went to Birmingham (Ulva, diatoms), Newcastle (barnacles) and Den Helder (seawater bacteria) to (try to) perform some of the biofouling evaluations myself, although I must say that few of those results ended up in the paper. The assays performed by the professional evaluators clearly showed that the hydrogel decreased the settlement rate of all tested organisms, compared with the unmodified glass substrate. Glass, of course, is not what ships are made of, but it seemed to be a reasonable standard to use as a comparison without complicating the study with too many variables. The organism that modified its behavior the least was probably the alga Ulva, which grew relatively well on the surfaces, although it settled in low densities. Barnacles did not settle to any major extent (not at all, in some of the assays performed) and the diatoms and bacteria did not seem to attach well to the hydrogel. We also studied the stability of the hydrogel surfaces. It appeared that no significant degradation took place over a 6-month period. This result is interesting, since oxidative degradation is seen as a major hurdle for the use of PEG in many applications. However, salt buildup in/on the material did take place, and this might be problematic for the long-term performance of these materials.
III Lateral control of protein adsorption on charged polymer gradients.

Ekblad T, Andersson O, Tai F-I, Ederth T and Liedberg B.


Contribution: Planned the work, fabricated all surfaces. Performed the IR measurements and took part in the SPR and AFM measurements. Wrote the manuscript, except the sections describing iSPR and AFM.

Description and comments: I attended a talk held by Shaoyi Jiang at the AVS conference in 2007. He talked about the progress his group had made with zwitterionic materials as nonfouling surfaces. After returning home, I thought that I would try something along the same lines. Andréas Larsson had developed the technique to make hydrogel gradients for his PhD and I applied the same principles, but in a “layered” system with two differently charged polymers. The plan was that there would be a protein adsorption minimum somewhere along the gradient. The result was, for someone with some experience in science, surprisingly good, since the gradients actually behaved as predicted.

The SIPGP method allows deposition of polyelectrolytes in a very different manner compared with the commonly used self-assembly processes for similar systems (layer-by-layer techniques). In principle, a layer can be made as thin or thick as is required, since the amount of deposited material is not (primarily) governed by electrostatic interactions. So, instead of inversing the charge of the first layer, the second layer may either be too thin to significantly affect the charge, or thick enough to dominate the charge characteristics of the surface. Or somewhere in between, which was the main idea with these gradients. It is, admittedly, difficult to fabricate a globally protein resistant two-layered coating, with both layers at the exact right thicknesses throughout the surface. A gradient is convenient in this respect, since one can always be sure that the right conditions will appear somewhere along it.

We have continued the work with these gradients and other similar charge gradients and they always show a protein adsorption minimum as long as both polymers are charged. At low or high pH the minimum disappears (for weak polyelectrolytes at least). The minimum is not always zero, and it is likely that some proteins will adsorb even to a completely charge-balanced zone, if the proteins interact with the polymers on the surface through other forces than electrostatics, such as hydrogen bonding. However, the system demonstrated in the paper (at high ionic strength and neutral pH) was definitely highly resistant to protein adsorption. This implies that the oppositely charged groups must be situated very close to each other on the polymer surface, since a phase separated system, even on the microscale, would probably lead to protein adsorption.
IV   Blood compatibility of photographed hydrogel coatings.

Faxälv L, Ekblad T, Liedberg B and Lindahl TL.


*Contribution:* Equal contribution with L. Faxälv. Planned and performed the experiments and wrote the manuscript together with L. Faxälv.

V   Patterned hydrogels for controlled platelet adhesion from whole blood and plasma.

Ekblad T, Faxälv L, Andersson O, Wallmark N, Larsson A, Lindahl TL and Liedberg B.

*Submitted.*

*Contribution:* Equal contribution with L. Faxälv. Planned and performed the experiments together with L. Faxälv and O. Andersson. Wrote the manuscript together with L. Faxälv.

*Description and comments:* Both papers discuss the blood compatibility of grafted hydrogel coatings, and were planned and written together with Lars Faxälv at the division of Clinical Chemistry, Faculty of Health Sciences, Linköping University. In the first work, several hydrogel coatings with different chemical compositions were tested in parallel. They were characterized both in terms of their physicochemical properties and their performance in blood contact, particularly the coagulation response and platelet adhesion. The coagulation was monitored with a novel method developed by Lars Faxälv. A minor protein adsorption study was also carried out to assist the interpretation of the results. Based on the findings in Paper I, we expected low adsorption of serum and plasma on the P(PEGMA-co-HEMA) coating, but the results that indicated an apparent high blood compatibility of PHEMA were surprising to us. It appears that PEG segments were not necessary to produce a nonfouling surface. Similar results have been found for very dense PHEMA films prepared with ATRP, and this finding again indicated to us that the architecture of SIPGP-synthesized films is likely to be dense and cross-linked.

The second work is partly a follow-up of Paper I, since it deals with the same surface chemistry. The interactions with platelets were studied in detail, especially as a function of hydrogel thickness, and a critical minimum thickness was found. The hydrogel coating was inert to platelet adhesion, but functionalization with appropriate surface-bound proteins made it possible to bind platelets specifically to the surface. The proteins were immobilized in patterns, and two different patterning methods were demonstrated. This system could be applied as a platelet function assay, since non-specific adsorption of blood proteins, which would result in uncontrolled platelet adhesion, can be avoided. We used imaging SPR to study the platelet adhesion, and to the best of our knowledge this is the first time that technique has been used in this way.
VI  Swelling of grafted poly(ethylene glycol)-containing hydrogels – a neutron reflectivity study.

Ederth T and Ekblad T.

In manuscript.

Contribution: Fabricated all hydrogel samples and took part in the neutron reflectivity measurements. Did not contribute significantly to the data analysis or to the writing.

Description and comments: The materials formed by the SIPGP method are, as noted previously, not particularly easy to characterize in terms of molecular architecture. We therefore seized upon an opportunity to use neutron reflectometry (NR) to characterize the hydrogel coatings. Thomas Ederth, who has worked with the method previously, applied for and received some beam time at ILL in Grenoble. I came along to make the coatings, help out with the long measurements, and learn something new. As mentioned above, NR allows simultaneous determination of the film thickness and the density profile, and is a suitable method for characterization of solid-water interfaces. This therefore appeared to be the ideal technique for answering a critical question regarding the hydrogel structure, namely, whether the degree of cross-linking and the grafting density varies along the direction normal to the surface. It may well be the case that “older” grafted polymer chains closest to the substrate surface are more cross-linked and branched than the “newer” chains, formed at the end of the reaction.

Unfortunately, we had some problems with our measurements. The reactor was emergency stopped due to a malfunctioning filter, and then it took a few days to restart it. We were fortunate enough to get another application for beam time at a later date approved, but the data from that experiment (which was carried out on a different instrument, D17) has not yet been thoroughly analyzed. Therefore, the manuscript included here will most likely undergo some changes before submission for publication. We mainly studied how hydrogels swell in humid air and in water. Although the swelling experiments in humid air are relatively conclusive, the experiments in water proved quite difficult to analyze. However, it appears that hydrogels prepared on different substrates (gold and silicon) have slightly different density profiles. This is an important result, as we have often used both types of substrates in combination to facilitate convenient characterization. That approach should apparently be used with some care.
VII  Novel application of imaging surface plasmon resonance for *in situ* studies of the surface exploration of marine organisms.

Andersson O, Ekblad T, Aldred N, Clare AS and Liedberg B


Contribution: Equal contribution with O. Andersson. Planned and performed the experiments together with O. Andersson and N. Aldred. Contributed to the writing of the manuscript.

VIII  *In situ* quantification of surface exploration and footprint deposition by barnacle cyprids (*Semibalanus balanoides*) using imaging surface plasmon resonance.

Aldred N, Ekblad T, Andersson O, Liedberg B and Clare AS.

*In manuscript.*

Contribution: Fabricated the surfaces. Performed the experiments together with O. Andersson and N. Aldred. Performed much of the data analysis.

Description and comments: Both papers describe the use of imaging surface plasmon resonance for the study of barnacle cyprids. This is definitely a new application of that technique, and it appears to have potential to become quite useful. The work was initiated after discussions between me, Bo and Nick Aldred at a poster session at a joint AMBIO/ONR workshop. Nick has previously studied cyprid behavior and cyprid footprints using many different techniques. The study of cyprids is critical for furthering the understanding of how antifouling coatings can be designed, since the barnacle decides where to settle permanently at that stage in its life cycle.

The extreme surface sensitivity of iSPR makes it possible to study events on the surface or in direct vicinity to it. As a cyprid “walks” over the surface it only seems to touch it with its two antennules, yielding a clear trail of “steps”. The cyprid may, or may not, deposit material (a “footprint”) upon contact with the surface. The fact that this process can be analyzed with iSPR was the main message of Paper VII. Much of the contents of the subsequent Paper VIII describe how the rate of footprint deposition can be quantified and how this quantity differs for different surfaces. We found some clear differences between surface chemistries, with the poly(PEGMA-co-HEMA) hydrogel coating showing the lowest rate of footprint deposition.

There is still plenty of room for improvement of the iSPR method. The instrument was not originally built for this purpose, and the design may be optimized for the application. For instance, the area visualized in the current iSPR setup is too small to continuously track the activities of a single organism, since the cyprids tend to pass through the field of view rather rapidly. This work will continue, hopefully leading to a mature and useful technique for biofouling-related studies of cyprids, and perhaps also of other organisms.
6 CONCLUDING REMARKS AND FUTURE OUTLOOK

The work described in this thesis has had a relatively broad focus in terms of applications, but was held together by a common theme: the pursuit of nonfouling materials. The contribution of this work in relation to the wealth of literature and studies already devoted to the same subject is perhaps modest, but I would like to point out a few important findings, which I hope will find uses in the future.

- A method to prepare thin, surface-grafted hydrogel films bound to the surface of plastic materials was developed. This method does not require any prior treatment of the substrate and is capable of producing functional coatings with excellent properties, particularly when low protein adsorption is required.

- A specific use of the method is the grafting of polyelectrolytes to surfaces. The thickness of the deposited layer(s) can be easily varied along a gradient or in patterns. Polyelectrolyte bilayers may become protein resistant, provided that the ratio of positive and negative charges is balanced.

- The results in Paper II provide clear support to the hypothesis that there is a connection between low protein adsorption and the settlement rate of several of the most important organisms responsible for marine biofouling. The main challenge is to keep the adsorption of protein low over time.

- Surface plasmon resonance is a valuable tool not only for biomolecular interaction analysis but also, in the imaging format, for analysis of the surface-related behavior of cells and higher organisms, specifically barnacle cyprids.

The first two points relate to the SIGP method, which was already described but not fully developed or utilized for biological applications before the start of this work. So what does the future hold for this method? So far the academic interest has been limited, although the last year has seen an increased number of publications (from a very low number). Perhaps uses will be found in niche applications, especially for the modification of flat plastic substrates, such as the bioanalytical devices discussed here. The macroscopic properties of the coatings appear to match those of comparable coatings prepared with more well-controlled methods, and as long as the lack of control of the microscopic architecture can be tolerated, the simplicity of the method is a clear advantage. In addition, the swiftness and versatility of the method could allow it to be used as a primary step to functionalize inert materials, prior to further (if necessary, more controlled) modifications. It would, however, be desirable to better determine the resulting polymeric microstructure and how it is affected by variations in different reaction parameters.
The use of imaging SPR for analysis of the behavior of cells and organisms was shown in Paper V and Papers VII-VIII, respectively. In Paper V, blood platelets were studied with iSPR, yielding the interesting result that only cells which interacted with the surface through receptor-ligand binding could be observed, while all other cells remained invisible (i.e., they were outside the penetration depth of the technique). This effect may be quite useful for analyzing general cell-ligand interactions, and deserves to be studied further. When applied to barnacle cyprids, the iSPR method appears to be capable of visualizing and quantifying events which were previously not experimentally accessible. It remains to be seen whether this method will deliver on its promises. If it does, it might become a very valuable tool for the design of novel antifouling coatings.

This thesis has merely touched upon the huge and interdisciplinary subject of marine biofouling; an area of research in which many discoveries and many ideas still remain to be explored. Perhaps it will be necessary to use several approaches together, each aimed at one particular target organism, to create persistently nonfouling non-toxic coatings. As our collective knowledge concerning the different organisms responsible for biofouling increases, we will most likely find new ways to deter them more efficiently, without poisoning the surrounding marine environment. This work has hopefully contributed with a step or two towards that goal.
7 REFERENCES


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