Biomimetic surfaces

preparation, characterization and application

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The cover mimics the substrates used in this thesis, which consist of silicon with a thin layer of gold on top.

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Abstract

This thesis describes the preparation, characterization and application of a few biomimetic surfaces. Biomimetics is a modern development of the ancient Greek concept of mimesis, i.e. man-made imitation of nature. The emphasis has been on the preparation and characterization of two types of model systems with properties inspired by nature with future applications in bioanalysis, biosensors and antifreeze materials. One type of model system involves phosphorylated surfaces; the other consists of surfaces mimicking antifreeze (glyco)proteins. The surfaces were made by chemisorbing organosulfur substances to a gold surface into monomolecular layers, so called self-assembled monolayers (SAMs). The physicochemical properties of the SAMs were thoroughly characterized with null ellipsometry, contact angle goniometry, x-ray photoelectron spectroscopy and infrared spectroscopy prior to application.

The work on antifreeze surfaces was inspired by the structural properties of antifreeze (glyco)proteins, which can be found in polar fish. Two model systems were developed and studied with respect to ice nucleation of condensed water layers. One was designed to mimic the active domain of antifreeze glycoproteins (AFGP) and the other mimicked type I antifreeze proteins (AFP I). Subsequent ice nucleation studies showed a significant difference between the AFGP model and a (OH/CH_3) reference system displaying identical wetting properties, whereas the AFP I model was indistinguishable from the reference system.

The model systems with phosphorylated surfaces were inspired from phosphorylation and biomineralization. Two systems were developed, short- and long-chained amino acid analogues, with and without a phosphate group. A novel approach with protected phosphate groups before attachment to gold were developed for the long-chained analogues. The protective groups could be removed successfully after assembly. The long-chained SAMs were evaluated with electrochemical methods and significantly higher capacitance values were observed for the phosphorylated SAMs compared to the non-phosphorylated.

Abstract (Swedish)

I denna avhandling beskrivs tillverkning, karaktärisering och tillämpning av ett antal biomimetiska ytor. Biomimetik är att härma naturen och grundtanken är att titta på hur naturen löst liknande problemställningar. Två olika typer av modellsystem med inspiration från naturen har tagits fram för framtida tillämpningar inom bioanalys, biosensorer samt antifrysmaterial. Det ena typen av modellsystem innefattar fosforylerade ytor och det andra består av ytor som härmar antifrys(glyko)proteiner. Ytorna tillverkades av monolager av självorganiserande svavelorganiska molekyler och karaktäriserades före tillämpning med hjälp av ellipsometri, IR-spektroskopi, kontaktvinkelmätning och röntgenfotoelektronspektroskopi.

Modellsystemen för att studera vattenfrysning på ytor inspi rerades av antifrys(glyko)proteiner som bl.a. kan hittas i polarfiskar. Två modellsystem utvecklades och studerades med avseende på frysning av kondenserat vatten. Det ena designades att härma den aktiva domänen hos ett antifrysglykoproteiner (AFGP) och det andra härmade typ I antifrysproteiner (AFP I). Frysstudierna visade på signifi-kanta skillnader för AFGP-modellen jämfört med ett (OH/CH_3) referenssystem med jämförbar vätbarhet, men inte för AFP I-modellen. Vattnet frös vid högre temperatur för AFGP-modellen.

Modellsystemen med fosforylerade ytor inspirerades av fosforylering och biomineralisering. Två system utvecklades, ett med långa och ett med korta alkylkedjor på aminosyraanalogerna, både med och utan fosfatgrupp. En ny metod användes med skyddsgrupper på fosfaterna hos de långa analogerna innan bildandet av monolager. Skyddsgrupperna togs bort efter bildandet av monolager. Dessa monolager undersöktes också med elektrokemiska metoder och signifikant högre kapacitans observerades för de fosforylerade monolageren jämfört med de icke fosforylerade.

Preface

The aim of the Ph.D. studies is to produce a capable new researcher by training the student in scientific and academic tradition. To accomplish this, my studies have consisted in a mixture of teaching, taking courses and performing research. This book—my thesis—summarizes the results of my research in five papers of which four are already published and one is in the form of a manuscript. They are preceded by introductory chapters that are intended to give an insight into the different fields that inspired and were used in the experimental work involved in my research. A summary of the papers is also included.

This thesis is focused on the study of thin molecular films on gold and is based on experimental work performed at the division of Molecular Physics, Department of Physics, Chemistry and Biology at Linköping University. I have been a graduate student within Forum Scientium, a multidisciplinary graduate school. It promotes thematic work and exchange between disciplines by letting students with different backgrounds shed light on different aspects of a common research problem. This has also been encouraged by the research group, Molecular Physics. Here cross-disciplinary research is done at the interface of surface analysis, surface chemistry, organic chemistry, biochemistry and sensor technology and this environment is a valuable asset in a project like this. An especially fruitful collaboration has been with the division of Organic Chemistry. They have shown me the importance of properly designed building blocks/molecules and made it possible to realize the model systems with molecules not available commercially.

Taking courses and teaching have also been interesting and I learned a lot but the real challenge was the research. Experiments that were not repeatable could lead to such bewilderment and frustration when it was difficult to deduce what the extra variable was that I had not accounted for. Broken or malfunctioning equipment have also been a source of exasperation. On the other hand, the research also led to such exhilaration and eagerness to do more measurements when a missing variable was deduced or I

got new inspiring ideas that I just had to test. My PhD studies have really been a roller-coaster and I would like to thank all of you who made this thesis possible and I hope you know who you are! I won't mention any names but none is forgotten.

Annika Borgh

November 2006

During the course of the research underlying this thesis Annika Borgh was enrolled in Forum Scientium, a multidisciplinary graduate school founded by the Swedish Foundation for Strategic Research, SSF and Linköpings universitet, Sweden. The research was sponsored by BIOMICS which was funded by SSF.

Publications

The thesis is based on the following papers:

I "A new route to the formation of biomimetic phosphate assemblies on gold: synthesis and characterization"
A. Borgh, J. Ekeroth, R.M. Petoral Jr., K. Uvdal, P. Konradsson, and B. Liedberg
Journal of Colloid and Interface Science 295, 2006, 41-49

II "Electrochemical evaluation of the interfacial capacitance upon phosphorylation of amino acid analogue molecular films"
J. Ekeroth, F. Björefors, A. Borgh, I. Lundström, B. Liedberg, and P. Konradsson
Analytical Chemistry 2001, 73, 4463-4468

III "Synthesis and monolayer characterization of phosphorylated amino acid analoges"
J. Ekeroth, A. Borgh, P. Konradsson, and B. Liedberg
Journal of Colloid and Interface Science, 254, 2002, 322-330

IV "Mimicking the properties of antifreeze glycoproteins: synthesis and characterization of a model system for ice nucleation and antifreeze studies"
A. Borgh and B. Liedberg
M Hederos and P. Konradsson
Journal of Physical Chemistry B 109, 2005, 15849-15859

V "Biomimetic surfaces for ice interaction studies"
A. Borgh, M. Östblom, and B. Liedberg
in manuscript

My contributions to the papers in the thesis:

I I planned, performed and evaluated the experimental work. Rodrigo Petoral Jr. performed some of the XPS measurements and contributed to their evaluation. I did all the writing. Johan Ekeroth did the synthesis of the thiols.

II Johan Ekeroth and I shared the planning, performing and evaluating the experimental work. Fredrik Björefors was responsible for the electrochemistry. I took part in the writing.

III I did a major part of the planning and performing of the experimental work. Johan Ekeroth shared the responsibility for the evaluation. I took part in the writing. Johan Ekeroth did the synthesis of the thiols.

IV I was responsible for the planning, performing and evaluating of the experimental work. Markus Hederos contributed. I was responsible for the design of the setup for measuring the crystallization temperature. I was responsible for the writing. Markus Hederos did the synthesis of the thiols.

V I was responsible for planning, performing and evaluating the experimental work. Mattias Östblom performed the measurements on water deposition in ultra high vacuum and contributed to their evaluation. I did all the writing. Johan Ekeroth did the synthesis of the thiols.

Glossary and abbreviations

Adventitious	Not inherent, inherited or innate but rather occurring accidentally or spontaneously.
AF(G)P	Generic term for AFP and AFGP
AFGP	Antifreeze glycoprotein
AFP	Antifreeze protein
Amphiphilic	'Loving both'; a chemical compound having both hydrophobic and hydrophilic nature.
ATP	Adenosine triphosphate
Capacitance	Capacitance exists between any two conductors insulated from another and is a measure of electric charge separated, or stored, for a given electric potential.
Cation	A positively charged ion.
Chemisorption	A type of adsorption whereby a molecule adheres to a surface through the formation of a chemical bond.
Colligative	A property of the solution that depends on the mole fraction of solute particles present but not on the molecular mass or chemical properties of those particles. When a solute is present, there is an additional contribution to the entropy of the liquid which opposes the tendency to freeze. Consequently, a lower temperature must be reached before equilibrium between solid and solution is achieved.
Cytoskeleton	The internal 'skeleton' of cells.
Glycoprotein	A macromolecule composed of a protein and a carbohydrate.
Goniometer	An instrument that either measures angles or allows an object to be rotated to a precise angular position.
HA	Hydroxyapatite, mineral found in bone and teeth.

Heterogeneous catalysis	This involves the use of a catalyst in a different phase from the reactants. Typical examples involve a solid catalyst with the reactants as either liquids or gases.
Hydrophilic	Water preferring.
Hydrophobic	Water rejecting.
Hydroxyl	-OH
IRAS	Infrared reflection absorption spectroscopy
Lyophilic	Solvent preferring.
Lyophobic	Solvent rejecting.
Methylene	$-CH_2-$
Methyl	$-CH_3$
Mole fraction	or molar fraction is the relative proportion of molecules belonging to the component to those in the mixture by number of molecules.
Nucleation	The onset of a phase transition, for example, the formation of liquid drops in saturated vapor or a crystal from a liquid.
Placoid	Platelike, hard toothlike scale of a shark.
Polyelectrolyte	Polymer whose repeating units bear an electrolyte (ionizable) group.
SAM	Self-assembled monolayer
Teleost fish	A fish of the subclass Teleostei, a large group of fish with bony skeletons, including most common fishes. The teleosts are distinct from the cartilaginous fishes such as shark, rays and skates.
Tribology	The science and technology of interacting surfaces in relative motion and all practices related thereto, e.g. friction, lubrication and wear. Derived from the Greek *tribo* meaning “I rub”.
XPS	X-ray photoelectron spectroscopy

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Introduction

The surface of a material is interacting with the surroundings and can be of vital importance for the function of an object. It can therefore be of interest to modify the surface to change its properties. Well-known examples of surface modifications are gold plating, frosted glass or a non-stick pan. Gold plating, for example, is done since a solid gold piece would in most cases be too expensive and in some cases too heavy or too malleable. By covering the item with a thin layer of gold it is possible to give the object the desired bulk characteristics while still benefiting from the surface characteristics of gold, such as the chemical inertness and color.

Surface science is a broad area of research including the preparation and characterization of a wide range of surfaces, and is of importance in many technological fields. Extensive research, both theoretical and experimental, is undertaken on fundamental surface phenomena like wetting, heterogeneous catalysis and tribology but also on the exploration of new applications. One strategy to design new surfaces is to use molecular thin films to control the interfacial properties. This has been used in many different fields from fundamental research on adhesion, wetting, etc, to applications such as biomaterials, biosensors, chemical sensors, and microelectronic devices.

The overall purpose of the work described in this thesis was to explore the potential of a biomimetic approach to designing molecular thin films for the creation of two different

kinds of surfaces for future use in both fundamental studies and applications such as bioanalysis, biosenors and anti-freeze materials. The concept “biomimetic” means imitating, copying, or learning from biological systems and the design of the two model systems was inspired by phenomena found in nature, see figure 1. One model involved phosphate groups and the other functional groups found to be of importance in certain antifreeze (glyco)proteins.

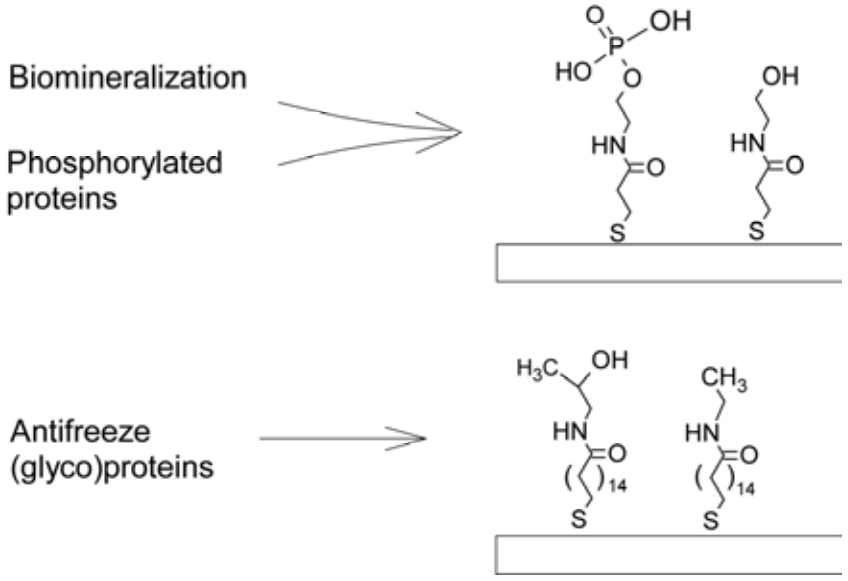

Fig. 1. Inspiration to my projects and some of the molecules used to realize the two different types of biomimetic surface designs investigated in my research.

Protein phosphorylation is the process where a phosphate group is attached to a serine, threonine or tyrosine residue on a protein. The phosphate group affects the protein function, for example, in enzymes the phosphate group can function as an on/off switch. Phosphorylation is a key event in many biological processes such as the metabolism, the organization of the cytoskeleton, signal transduction, cell cycle control and gene regulation. Designed, biomimetic

surfaces would allow for bioanalysis of phosphorylation/dephosphorylation activities as well as water and metal ion coordination to phosphorylated amino acids. There is also the possibility of developing these surfaces further into sensing elements in sensors for metal ions and phosphorylation/dephosphorylation activities. The biomimetic surfaces are also relevant for the creation of soft templates for biomineralization which could be used to improve the integration of bone prostheses.

An antifreeze (glyco)protein, AF(G)P lowers the freezing temperature of a solution by binding to ice seed crystals and preventing further growth. This interaction is not well understood and a biomimetic surface would give opportunities to fundamental studies of the interaction between ice and organic/biological molecules. Surfaces with controlled ice nucleation, at a higher or lower temperature than normal, have many applications. A surface designed specifically to induce ice nucleation at a relatively high temperature would increase the possibility to get good arena ice surfaces for ice-skating, curling or hockey. Lowering the ice nucleation temperature could instead be of use for antifreeze materials on airplanes, bridges, power lines or the windshield of a car. Ice on an airplane affects the aerodynamics of the plane, and it poses a serious security hazard. Because of this, airplanes are deiced before take-off using large amounts of deicing agents, which is a costly, time-consuming and environmentally problematic procedure.

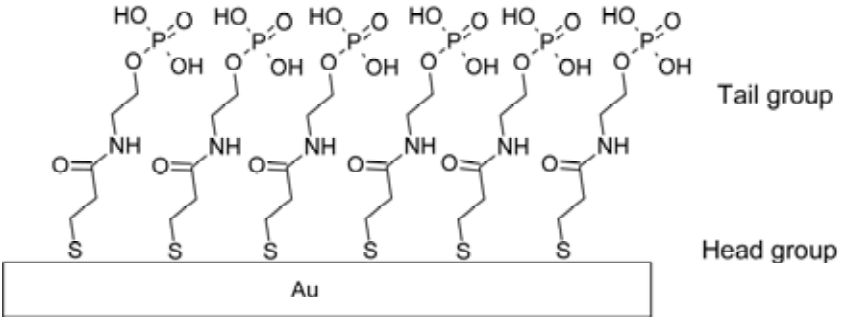

Fig. 2. Schematic illustration of a phosphorylated SAM on gold with tail groups exposed at the vacuum/ambient interface.

The model systems were created from self-assembled monolayers, SAMs, of alkanethiols on gold substrates. A SAM is a two-dimensional film, one molecule thick, which self-assembles with the head groups bound to the substrate and thus exposes the tail groups at the vacuum/ambient interface, see figure 2. A wide range of functional groups can be introduced into the SAMs making it possible to tailor and optimize the properties of the surface; the SAMs are also easy to prepare and reproduce.

The biomimetic models were characterized with standard surface science techniques. Contact angle goniometry is a surface sensitive method based on liquids to probe the outer part of the SAM. Ellipsometry was used to measure the thickness of the SAM and the composition of the monolayer was determined with X-ray photoelectron spectroscopy, XPS, and infrared spectroscopy. Infrared spectroscopy was also used to study the functional groups in the samples to ascertain that the desired interfaces were obtained.

Biomimetics

Mimicking is something all humans do and it is important for our development. During our first years we copy our parents' words when we are learning to speak. But children do not limit themselves to mimicking speech, and I think most parents have realized that "children do as we do, not as we say". Humans are, however, not limited to just mimicking other humans; nature is a wonderful source of inspiration to learn new things from. Birds, for example, have for a long time inspired man into trying to fly. Already in Greek mythology there is an example of humans constructing artificial wings. Daedalus was trapped on the island of Crete, and so he built wings out of feathers and wax for himself and his son, Icarus. They left the island, but Icarus flew too close to the sun, so the wax melted and he plunged into the sea and died. The models have fortunately improved, and nowadays it is common for humans to fly.

Evolution has had billions of years to come up with extraordinary materials and concepts to solve problems that also face humans, and nature has been a source of inspiration, both for function and aesthetically, in a variety of fields [1-8]. Today when a process, substance, device, or system is developed to copy or imitate nature it is often called biomimetic [7-13]. Precisely what is included in the term biomimetic can, however, differ. The most common definition involves extracting principles from biology and applying them to man-made devices, but the term can also include copying

biomolecular building blocks and applying them for new purposes.

Chitin, see figure 3, is one example where new uses for a biomolecule have been found [7, 14, 15]. This polysaccharide can be found in algae, the cell walls of fungi, the exoskeletons of arthropods, etc and is one of the most abundant organic compounds on earth with a production that is estimated to be around 10^{11} tons each year. Chitin and its deacetylated form, chitosan, have several interesting properties; they are, among other things, non-toxic, non-allergenic, anti-microbial, and biodegradable, they have fiber- and film-forming properties and they adsorb metal ions. Chitosan is also suitable as a substrate for biomimetic polymers because the free amino groups in the polymer backbone are easily modified. In the medical field chitin and its derivatives have been used for drug delivery, contact lenses, wound dressing materials, suture materials, and tissue engineering, but they are also used in fields like cosmetics, textiles, nutritional supplements, water purification, foods and agriculture.

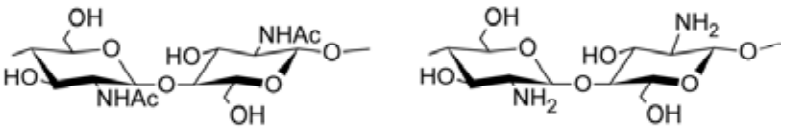

Fig. 3. New applications have been found for the biomolecule chitin (left) and its deacetylated form, chitosan (right).

Robotics is one field where research is being done to extract principles from biology and apply them to human-made devices [6, 13]. The physical construction and mechanical design principles of the cockroach has, for example, inspired the design of a robust six-legged robot [16]. Another robot inspired by insect locomotion also had a tracking system based on the cricket auditory localization [17]. The robot had auditory circuits closely based on the cricket ears and was controlled by a neural network mimicking the known neural network in the cricket.

Biomimetic approaches are also useful in surface science; biology has, for example, guided researchers to a new strategy for the creation of self-cleaning surfaces [8, 18-20]. Non-wettable plant leafs, such as those of the lotus plant, have a built-in cleaning mechanism where contaminants (dust, spores etc) are removed by rain, fog or dew [21, 22]. The surface of the Lotus leaf is ultrahydrophobic, with a contact angle of 162°, caused by a combination of surface roughness and hydrophobic properties of the surface material, see figure 4. On such rough low-energy surfaces, water gains very little energy through adsorption to compensate for any enlargement of its surface and thus forms an almost spherical droplet on top of the surface structure. The hysteresis in contact angle is small, typically 5-10°, which is important for a self-cleaning surface since the pinning force that has to be overcome by external forces, wind, gravity, etc is related to the hysteresis [18, 23]. If it is too large, the drop will stick or be smeared out across the surface. The particles deposited on the leaf are also larger than the surface microstructures and consists, in most cases, of materials that are more readily wetted than the surface of the leaf. As a result the contaminating particles are picked up by water droplets and then removed with the droplets as they roll off the leaves; this is sometimes called the Lotus-effect. There is a broad area of possible applications for ultrahydrophobic surfaces ranging from the high-tech field of microfluidic applications in biotechnology to self-cleaning window glass in the household-commodity sector [18, 24].

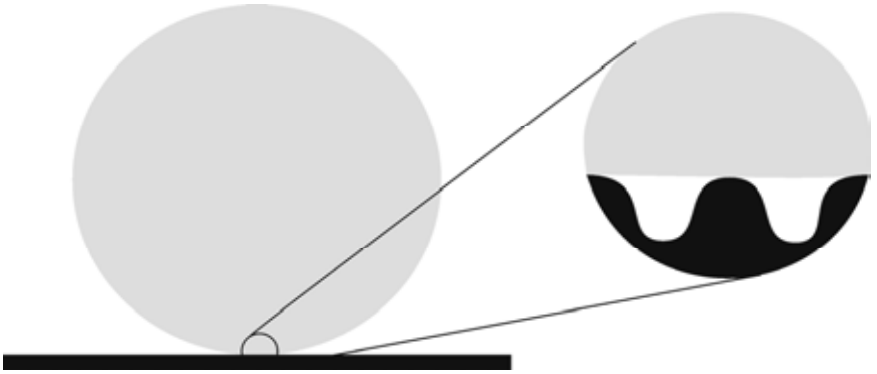

Fig. 4. Schematic drawing of a water droplet on the ultrahydrophobic surface of a Lotus leaf.

Biomimetic designs where surface roughness influences the wettability are also utilized when trying to solve the problems of biofouling [23]. Much effort is put into minimizing settlement of marine organisms on boats, ports and anything kept in the sea for a period of time [25, 26]. Traditionally anti-fouling paints containing heavy metals and herbicides have been used, but they contribute to marine pollution and harm a variety of sea life. As an alternative, biomimetic-inspired approaches are being explored [26, 27]. The Sharklet AF™ design is based upon the configuration of placoids of fast moving sharks. This design is an engineered microtopography, combining surface roughness and a hydrophobic surface and it has been demonstrated that it inhibits the settlement of spores of a marine alga [27].

Biomineralization

Biomineralization encompasses the study of the formation, structures and properties of inorganic solids deposited in biological systems [28]. Organic architectures in association with inorganic solids give unique and exquisite biominerals in biological organisms. Bone, tooth, coral, ivory, pearls are just a few examples of a wide variety of biomineralized materials with properties that man find it difficult to duplicate. All these biological tissues are in addition synthesized in aqueous environments under mild physiological conditions,

which are not the case for many of the man-made inorganic materials, e.g. semiconductors. Biomineralization has, for example, inspired many researchers in the development of new materials and new manufacturing methods for ceramic components, biomedical devices, semiconductors, optical and electronic films, sensory devices and composite materials [1, 3, 7, 8, 10, 12, 29-32].

Biomineralization takes place on the cell wall, in the spaces between closely packed cells, inside intracellular compartments, or extracellularly on or within an insoluble macromolecular framework. It can be divided into two categories, biologically induced and biologically controlled mineralization [12, 28]. In biologically induced mineralization, the deposition is largely adventitious. Although the organic components (lipids, proteins and polysaccharides) can influence the nucleation process, little control is exerted over the growth of the mineral, which is usually formed along the surface of a living cell, where it remains firmly attached to the cell wall. The size, composition, shape and organization of the mineral particles are often poorly defined and heterogeneous. One example in this biomineralization category is ice nucleation caused by aggregated proteins in the cell wall of the bacterium *Pseudomonas syringae* [33, 34]. The proteins serve as a template to assemble the water molecules and it is speculated that threonine residues are involved. This ability has several potential applications, for example, in production and texturing of frozen food, cloud seeding and cryopreservation. It is actually already utilized in a product called Snomax®, consisting of a powdered form of freeze-dried bacteria, which is mixed in water for making snow at ski resorts [35, 36].

The other biomineralization category is the biologically controlled mineralization that organisms use to synthesize organic-inorganic composites to generate structural support, protection, gravity sensors, etc [12, 28]. The resulting biominerals from these highly regulated processes have reproducible and specific properties, including particle sizes, shape, composition, texture, and preferential crystallographic orientation. They have high levels of spatial organization and in many cases complex morphologies are ordered

in hierarchical structures at several length scales. Materials with exceptional chemical, mechanical and electrical properties are derived from the fine composite structures, for example, several thousands times as tough as the pure minerals of which they are composed. However, the details about the molecular interactions governing their construction are still largely unknown. Using organic assemblies such as oil/water emulsions, micelles and gels, chemists have developed synthesis strategies for inorganic materials with some control over the morphologies [12, 29, 30, 37]. More elaborate schemes are, however, needed to gain more control and understanding of the mineralization process.

One important aspect in biomineralization is the interface between the organic phase and the inorganic phase and there is, for example, ongoing research focused on controlled nucleation and growth of inorganic compounds on surfaces [12, 31, 32, 38]. Knowledge of how to grow crystals with a certain orientation and size at specific locations would make it possible to produce new ceramics, semiconductors and electro-optic materials. Self-assembled monolayers (SAMs) on metal substrates offer organized organic surfaces with possibility to choose chemical functionalities, as well as intermolecular spacing, ordering and orientation, and they are excellently suited to control oriented nucleation of crystals. Aizenberg et al. have used SAMs of alkanethiols with different functional tail groups on gold and silver to study calcium carbonate nucleation [39]. They found that different functional groups on different substrates induced calcite nucleation from specific crystallographic planes. They also made two-dimensional arrays of single calcite crystals by using patterned SAMs, and manufactured surfaces with a large number of indistinguishable nucleation regions, where one crystal was face-selectively nucleated, see figure 5 [40].

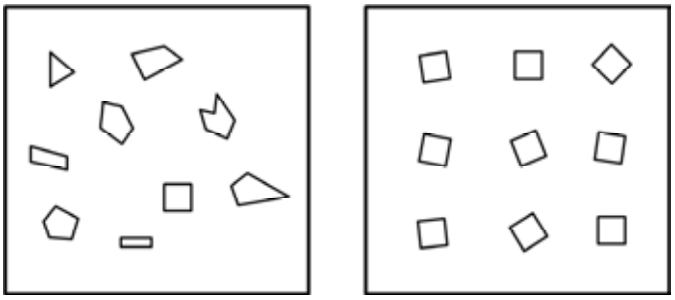

Fig. 5. Nucleation of minerals on surfaces: (left) non-oriented and (right) a two-dimensional array of single crystals formed by face-selective nucleation, i.e. the crystals are crystallographically oriented.

Extended polymeric networks are often associated with the construction of biominerals such as shell, invertebrate teeth, and siliceous plant material. The relatively inert structural frame, built from insoluble macromolecules, is accompanied with acidic proteins and the onset of mineralization seems to take place at the interface between the acidic proteins and the aqueous solution [12]. To explore this aspect, and in the hope of achieving more complex morphologies, SAMs on gold have been immersed in solutions of mineral ions and poly(acrylic acid), a polyelectrolyte, as a template for the nucleation of $CaCO_3$ [41] or $SrCO_3$ [42]. In both cases the authors observed ordered composite structures on the carboxyl-terminated SAMs. Long mineral wires were formed on the surfaces, with a length of more than 100 μm and a diameter of 250 nm ($CaCO_3$) or 150 nm ($SrCO_3$). They hypothesized that the organic backbone in the wires consisted of several hundreds of partially overlapping poly(acrylic acid) strands. Both the SAM and the poly(acrylic acid) were necessary in this cooperative process; no nanowires were formed if the poly(acrylic acid) or the SAM was excluded, or if the functional groups were changed. Another ordered mineral structure was formed on a surface in a different system based on hydroxyl-terminated SAMs, poly(aspartic acid) and $CaCO_3$ [43]. The combined interac-

tion of these components formed hollow vaterite spheres with diameters ranging between 2 and 5 μm.

Bone

Bone is a biocomposite that sometimes is called a living mineral because it undergoes continuous growth, dissolution and remodeling in response to both internal and external signals, for example pregnancy and gravity [37, 44]. Many defects, such as a broken bone, heal spontaneously with minimal treatment. But still, bones, or part of them, sometimes need to be replaced, due to damage by accidents, aging, or other causes. This has been done throughout history, and successful replacements with wood, coral, ivory, jade, animal bone, gold, sapphire, etc, have been found in fossil evidence from the civilizations in the Nile delta, Mesoamerica and later in Europe [44]. Some of these materials have similar structures as bone and are actually still of use as biomaterials [37]. Currently, a biomimetic approach is often used in the research focused on improving the biomaterials.

Bone and teeth consist of the mineral hydroxyapatite (HA) in combination with a large number of proteins, often phosphorylated at serine residues [12]. The mineral can be referred to as $Ca_{10}(PO_4)_6(OH)_2$ even though in reality it is not compositionally pure; it is often calcium-deficient and enriched in CO_3^{2-}, which replaces PO_4^{3-} ions at various lattice sites [28]. The mechanical properties of bone are derived from the organized mineralization of HA within an insoluble framework of cross-linked collagen fibrils. Around 200 soluble proteins are also involved in controlling nucleation, mineral growth and general cellular activities associated with biomineralization, such as ion transport, enzymatic regulation and hormonal signaling.

Bone is the second most common transplantation tissue and techniques to regenerate bone have become a major clinical need; only blood is replaced more frequently [37, 45]. Bone grafts are used for smaller replacements, and the most common surgical approach is to use grafts from other regions of the patient. These grafts have excellent fusion

rates but there are also disadvantages, such as the size of the graft being limited and the necessary surgery being expensive and painful. There are alternative grafts, such as donated bone (from cadavers), demineralised bone matrix, coral, or ceramics, but none have yet all the benefits of the patient's own bone [37, 45, 46]. For larger replacements, such as the hip joint, implants are used; see one example in figure 6. Because of the high load on the hip joint, metals, like titanium and its alloys, have been the primary materials, due to their superior mechanical properties [47]. The problem with the metal implants is the interface and integration with the bone, and most hip implants must be replaced after 15 years. This is a growing problem, since an increasing number of patients live longer than 15 years after their operation. The current trend is to use surface modifications to improve the life-time of the implant; often the strategy is to make a biomimetic surface layer.

Fig. 6. X-ray image showing hip replacement (to the left). (Image from the public domain created by the National Institutes of Health, part of the United States Department of Health and Human Services.)

One approach is to coat the implant with a bioactive layer that can promote the attachment of bone-forming cells and

the formation and adhesion of HA [31, 46-51]. A biologically active and bone-like apatite layer is believed to be the main requirement for the artificial material to bond to living bone [50]. The currently most used commercial method to achieve this is by using plasma spraying to produce a HA coating on the implant. The implants develop a strong connection with the bone tissue in a short time, but there are several concerns with this process [49-53]. One regards resorption and degradability of HA coatings in a biological environment, another is the poor bonding between the plasma sprayed HA coating and the titanium. This may lead to disintegration of the coating, resulting in the loss of both the coating-substrate bonding strength and implant fixation. The process is also carried out at high temperatures (>1000 °C) in a line-of-sight method, and there are questions regarding the long-term reliability. Heating increases cost and the probability of contamination, and it can have adverse effects on the mechanical properties of the substrate metal. These concerns are also valid for other thermal spraying techniques in commercial use. Other methods such as ion deposition, sol-gel coatings, chemical treatment with hydrogen peroxide or alkali, etc also experience these disadvantages to different degrees [12, 50, 53].

A biochemical approach has recently begun to be explored where the surface is modified in order as to induce HA nucleation, either when immersed in simulated body fluid containing Ca^{2+}, PO_4^{3-}, and OH^- ions before implantation or in the body after implantation. This involves the formation of a biomimetic surface with an ultra-thin layer of bioactive molecules that will reproduce the natural processes of biomineralization. Tanahashi et al. studied the growth of HA at low temperatures from a stimulated body fluid on SAMs with different functional groups on gold substrates [54]. They found that negatively charged surfaces act as potent substrates for HA nucleation, and the crystal growth rate on SAMs with different functional groups varied as: PO_4H_2 > COOH >> $CONH_2$ ≈ OH > NH_2 >> CH_3 ≈ 0. The most potent HA inducer was the phosphate-terminated SAM. In later studies, SAMs were used to introduce functional groups on titanium substrates [52, 53]. In one study HA was observed on both phosphate- and carboxyl-terminated SAM surfaces,

but phosphate-terminated SAMs showed a stronger nucleating ability [52]. However, in another study carboxyl-terminated SAMs were deemed to be superior to phosphate-terminated SAMs [53].

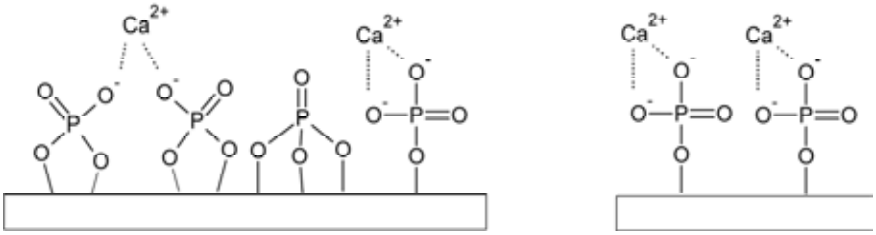

Fig. 7. Interaction modes between calcium and surface phosphate groups in the initial step for hydroxyapatite formation. To the left is a schematic illustration of the surface after phosphate groups are introduced by using $POCl_3$ on a hydroxyl SAM and to the right with the approach used in this thesis with SAMs formed from phosphorylated thiols. Note the difference in surface-molecule bond geometry.

Model surfaces

In the three above mentioned studies were the phosphate groups introduced via phosphorylation of hydroxyl-terminated SAMs by using phosphorous oxychloride ($POCl_3$). This leaves some uncertainty concerning the structure of the surface since this reaction may include the heterogeneous formation of phospho mono-, di-, and tri-esters, see figure 7 [55, 56]. The proximity of hydroxyl groups in a SAM actually makes the formation of di- and triesters very likely. A surface which truly has $-PO_4^{2-}$ tail groups could be a better HA inducer since it has been suggested that attraction of Ca^{2+} to a surface is an important initial step in HA formation [51]. To achieve better control over the structure of the phosphate groups on the surface, they can be introduced into the thiols before assembly. This strategy was used in the work described in this thesis, which details the characterization of SAMs of several phosphorylated amino acid analogues.

In conclusion, a bottom-up approach is often used in nature. Biological materials such as pearls, bone, silk, wood and muscle fibers are generated by assembling molecule by molecule into masterpieces of complex hierarchical structures that integrate many functions. The challenge in biomimetics is to elucidate the physical and chemical strategies used by nature in order to apply them, with suitable modifications, to the chosen application. The surface science work presented in this thesis was inspired by nature as I have tried to extract principles from biological structures and their functions and apply them to synthetic components. Two different biological phenomena mainly influenced the surface designs presented in this thesis, protein phosphorylation and antifreeze (glyco)proteins, but the models are also of interest from a biomineralization perspective. The phosphorylated surfaces are relevant to the study of phosphorylated proteins and kinase/phosphatase activity, but also as an interesting model system to study the nucleation of HA, as mentioned above. The antifreeze (glyco)proteins, AF(G)Ps, prevent polar fish from freezing in icy sea water and inspired models for the study of freezing at surfaces. The AF(G)Ps are sometimes included as a special case in biomineralization since the proteins interact with ice seed crystals. Knowledge of the mechanism behind their function could therefore perhaps be transferred to systems with more conventional molecules.

Protein phosphorylation

Proteins are essential to the structure and function of living cells and viruses. DNA may contain the instructions necessary for life, but it is the proteins that carry out the instructions. The word *protein* is actually derived by Jöns J. Berzelius in 1838 from the Greek word *proteios*, which means “of the first rank” to emphasize the importance of this class of molecules [57]. A protein is an organic macromolecule consisting of an unbranched chain of amino acids that adopts a defined three-dimensional structure, a conformation. The number of amino acids in an average protein is around 300, but the largest protein found so far consists of a single chain of nearly 27 000 amino acids [58]. Some examples of functional classes where proteins play key roles are enzymatic catalysis, mechanical support, control of growth and differentiation, transport and storage, defense, and signaling.

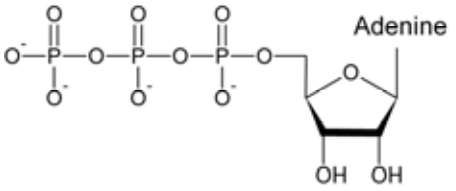

Fig. 8. Schematic structure of adenosine triphosphate, ATP, one of the nucleoside triphosphates.

Protein function is strictly regulated, and one of the most important regulatory mechanisms is reversible protein phosphorylation [57]. Fischer and Krebs received the Nobel Prize in Medicine in 1992 "for their discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism". Their research began in the 1950's when they showed that an enzyme could be converted from an inactive form by a mechanism involving the transfer of a phosphate group from adenosine triphosphate, ATP, see figure 8 [59]. This was the starting point for the study of phosphorylation in medical research, which has resulted in a large number of articles, the Medline database listed over 130 000 (1966 - June 2006).

When a protein is phosphorylated, the terminal phosphate group of a nucleosoide triphosphate, see figure 8, is transferred to an amino acid that contains a hydroxyl group, see figure 9. The enzymes catalyzing this covalent modification are called protein kinases.

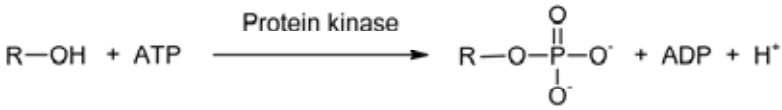

Fig. 9. Phosphorylation of an amino acid containing a hydroxyl group (-OH) by a protein kinase.

Protein phosphatases reverse the effect of kinases by catalyzing the removal of the attached phosphate group, see figure 10. The hydroxyl-containing amino acid is regenerated and orthophosphate is formed.

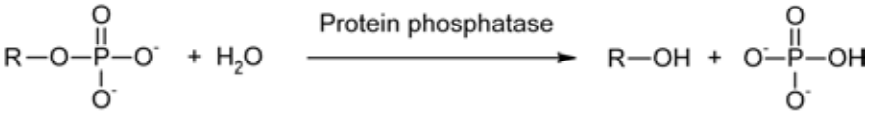

Fig. 10. Protein dephosphorylation of a phosphorylated amino acid by a protein phosphatase.

At first glance it might seem unlikely that the small phosphate group would have any impact at all on the much larger protein, unless it is directly involved by being part of or blocking the active site. There are, however, several other reasons why phosphorylation is an effective means to control protein activity. The phosphate group introduces several possibilities for interactions with other parts of the protein and the surrounding. It is very hydrophilic, adds two negative charges and can form three hydrogen bonds. The free energy of phosphorylation is also large and together this makes it possible for the phosphate group to change the characteristics of the protein, often by changing the conformation. The conformation of a protein is crucial for its function, and the reversible phosphorylation can thus function as an on-off switch, see figure 11. Some proteins can be phosphorylated on several amino acids [60].

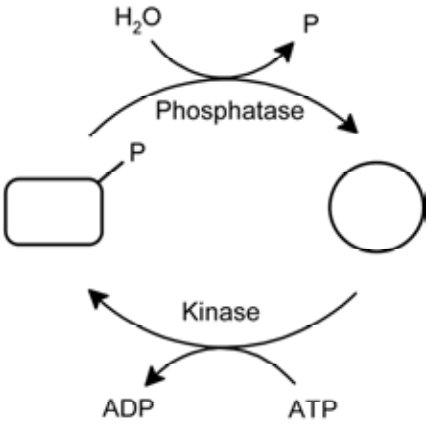

Fig. 11. Reversible protein phosphorylation. A protein kinase transfers a phosphate group (P) from ATP to the protein, which changes its biological properties. A protein phosphatase can remove the phosphate group. In this way the amount of phosphate that is associated with the protein is determined by the relative activities of the kinase and the phosphatase.

Today it is believed that a large portion (~30%) of the proteins present in a typical mammalian cell is subjected to phosphorylation; and abnormal phosphorylation is a cause or consequence of many diseases, including cancer, diabetes and chronic inflammation [61, 62]. Many naturally occurring toxins and pathogens also exert their effects by altering the phosphorylation states of intracellular proteins, the results are for example seafood poisoning and the bubonic plague.

Interaction studies

Traditionally, protein interaction studies have been performed in solution-phase assays, but this is partly changing as methods are being developed where one of the interacting partners is immobilized on a solid support [62-66]. The proteins or peptides are immobilized on substrates such as planar surfaces, microwells, or beads. The protein microarray assays allow the identification, quantification and inter-

action analysis of a large number of target proteins from a minute amount of sample. Protein microarrays are becoming an important research tool for life scientists and they also have a potential to be used in diagnostics [66-68].

Most existing strategies rely on a high-affinity binding between the interacting molecules, which normally works fine for protein-ligand or antigen-antibody interactions, but which makes it difficult to study enzyme-substrate reactions [65, 69]. However, several different arrays have been applied to identify substrates and determine activities for kinases and phosphatases [62, 63, 65, 66, 70]. The general principle is to immobilize the potential substrates on a microarray and then to introduce the enzyme in solution. The enzyme activity is then detected, either by indirect measurements of substrate phosphorylation using fluorescently labeled anti-phosphate antibodies or direct measurements of the incorporation of radioactive phosphorous using [γ^{32}]- or [γ^{33}]-ATP. Macbeath and Schreiber described one of the earliest kinase assays in a microarray format using isotopic labeling [71]. They used whole proteins, but small peptides have also been utilized [70]. Recently, phosphatase activity was analyzed using a small organic molecule containing a fluorescent moiety and a phosphate group for enzyme recognition, see figure 12 [69]. When the molecule is dephosphorylated it becomes fluorescent.

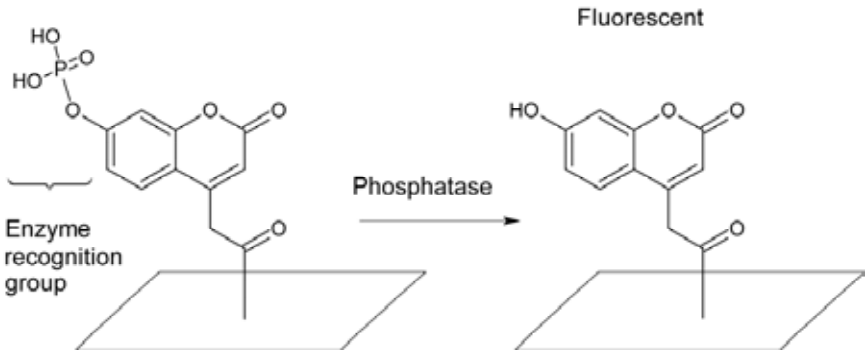

Fig. 12. Fluorescent strategy for detecting phosphatase. The phosphorylated molecule to the left is not fluorescent, but when phosphatase removes the phosphate group the molecule becomes fluorescent (right).

Self-assembled monolayers (SAMs) formed by alkanethiols on gold have also been used in arrays [63, 66, 72, 73]. Houseman *et al.* developed a peptide chip where the kinase substrates were immobilized on hydroxyl-terminated SAM surfaces via a Diels-Alder reaction [74]. The surfaces were incubated with a protein kinase and [γ^{32}]-ATP, washed, and the resulting phosphorylation was quantified using phosphorimaging. As an alternative to radioactive labeling the authors also used an antibody-based method to detect phosphorylation. Antibodies developed against phosphotyrosine were used in combination with surface plasmon resonance or fluorescence detection. However, this method does involve the modification of each peptide with a cyclopentadiene group, which makes it synthetically challenging [62].

There are, however, still several challenges associated with protein and peptide microarrays [63, 70, 73, 75]. One lies in the detection, fluorescent, colorimetric or radioactive labels are extensively used for detection, although a label-free method is preferable since labeling may alter the interaction [63, 66, 75]. Other issues include difficulties with achieving uniform labeling of proteins, software dependent readouts,

expensive instrumentation, and, not least, safety issues regarding radioactivity [62, 70, 73]. Also the specific antibody-based arrays for the analysis of protein phosphorylation have problems. One fundamental limitation is the problem with developing antibodies towards phosphorylated amino acids. There exist rather reliable antibodies towards phosphorylated tyrosine, but they still give rise to false negatives, and tyrosine phosphorylation is relatively rare [70, 76]. The commercially available anti-phosphothreonine and anti-phosphoserine antibodies give many false negatives and show cross-reactivity.

Model surfaces

A model system based on SAMs on gold is characterized in this thesis. There are several advantages with using SAMs on gold, for example, they are compatible with all of the principal techniques used for analyzing microarrays including phosphorimaging, surface plasmon resonance, fluorescence and matrix-associated laser desorption ionization mass spectrometry [70, 73]. Gold is also an electroactive substrate enabling electrochemical detection of surface properties and DNA arrays with electrochemical detection have been demonstrated [77, 78]. Electrochemical detection is sensitive, can be miniaturized, have large dynamic range, can give kinetic parameters, the instrumentation is inexpensive and the detection can be made label-free [63, 77].

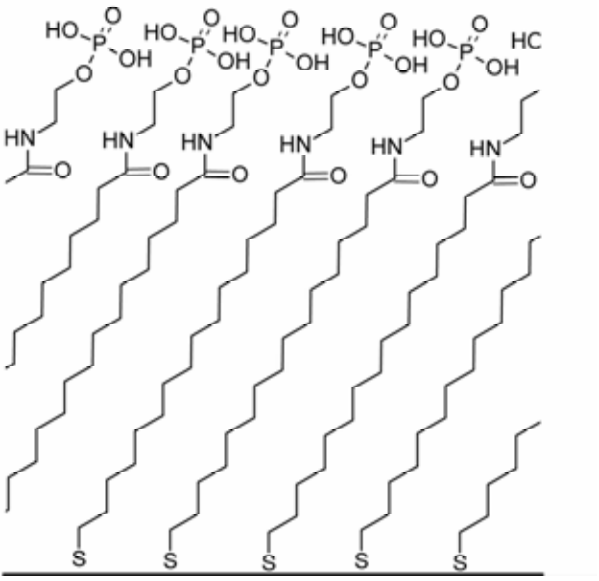

Fig. 13. Schematic illustration of a section of a phosphorylated SAM with long-chained serine analogues.

Serine, threonine and tyrosine thiol analogues with long alkyl chains, -$(CH_2)_{15}$-, were used to ensure that there would be a highly organized and insulating layer below the functional groups, an example can be seen in figure 13. Due to the amphiphilic nature of the phosphorylated analogues problems arose regarding the solubility and handling of the thiols. To avoid this, the thiols were assembled with phosphate protecting groups and deprotected in the SAM. Overall, a highly ordered assembly was observed for the non-phosphorylated analogues, but the protected phosphate, which was bulkier, disturbed the assembly to some extent, and the deprotection a little bit more, but well-ordered monolayers were still formed. It was possible to detect the difference between the phosphorylated and non-phosphorylated analogues by measuring the interfacial capacitance.

The results from the long chained analogues prompted an interest in trying to design phosphorylated analogues that

would not need protective groups. This was accomplished by using a short alkyl chain, $-(CH_2)_2-$. They formed well-oriented and densely packed monolayers on gold. The phosphate - counter ion interaction was studied by equilibrating a phosphorylated serine SAM with H^+, Na^+, Ca^{2+} and Mg^{2+} ions. Changes in IR-absorption for the outermost part of the molecule were seen indicating a change in the phosphate electronic structure for different counter ions, with magnesium ions showing the strongest coordination. The phosphate absorption in the presence of the divalent ions showed similarities to hydroxyapatite absorption.

Studies, not included in this thesis, have later been performed on the phosphorylated model system. For example, the influence of calcium and magnesium ion coordination on the interfacial capacitance was studied for both short- and long-chained phosphorylated serine analogues [79]. The short thiol formed monolayers with poor insulating properties, and the capacitance value for the SAM decreased significantly as Ca^{2+} or Mg^{2+} ions coordinated to the phosphates with the short alkyl chain. The introduced divalent ions are probably better at neutralizing the phosphate charges than the monovalent ions in the electrolyte thus lowering the polarity of the organophosphate layer and giving rise to the observed drop in capacitance. These results also indicated that the magnesium ion is the stronger phosphate coordinator. A smaller, insignificant, change was seen for the monolayers of long chains when calcium or magnesium ions were added to the solution. This is probably because the monolayer capacitance is dominated by the long alkyl chains and the influence from penetrating ions or solvent molecules into the monolayer is less because of the hydrophobic chains.

Another study also included aluminium and chromium ions on SAMs of short-chained tyrosine analogues with similar results as seen for the serine analogues [80]. Coordination of metal ions was, for example, observed to the phosphorylated analogue and not to the non-phosphorylated analogue. The presence of aluminium ions (Al^{3+}) resulted in the largest changes, including a change in the orientation of the phosphorylated analogues in the SAM.

In addition to affecting the polarity and charge of phosphates, calcium and magnesium ions are known to strongly promote fusion of vesicles and cells through coordination to the lipid head group. The SAMs terminated with phosphate groups have also been found to have potential as 2D templates for bivalent ion-mediated vesicle attachment and lipid bilayer formation [81]. Supported phospholipid bilayers and non-ruptured surface-bound vesicles are of interest as model systems of cell membranes, both for studying basic membrane processes and for possible biotechnological applications. Controlled formation of supported phosphatidylcholine bilayers or supported non-ruptured phosphatidylcholine vesicles could be demonstrated on SAMs of phosphorylated short serine analogues by varying the Mg^{2+} or Ca^{2+} content.

Another study investigated the interaction between water and the phosphorylated model system. SAMs of short serinephosphate analogues, with H^+, Na^+, and Ca^{2+} as counterions, and short serine analogues was studied [82]. This is of interest since water adopt special structures at interfaces, such as hydration shells around proteins and ions, and this interfacial water is believed to influence the interactions at the interface, for example, during mineralization. Thin layers, less than a full monolayer, of D_2O were deposited and a clear difference in the ice structure was observed with infrared spectroscopy for serine and serinephosphate SAMs. It was probably caused by the presence of the highly polar phosphate entity that possesses both hydrogen bond donating and accepting groups. These groups can readily replace the missing hydrogen bonds for the D_2O molecules in contact with the SAM, thus reducing the vibrations originating from non-coordinated D_2O bonds, i.e. surface bonds, in comparison with the serine SAMs. The D_2O ice layer was strongly bound to the serinphosphate surfaces with Na^+ and Ca^{2+} counterions, with D_2O-phosphate interactions stronger than D_2O-D_2O interactions. Weaker interactions were seen for the SAMs of serine and serinephosphate with H^+ ions, they showed almost identical behavior.

Antifreeze (glyco)protein

Ice is a material that sometimes is desirable and at other times or places it is most definitely unwanted. Ice is, for example, appreciated for cooling drinks, winter sports, and ice hotels, but icing causes trouble on aircrafts, ships and power lines. Ice is formed when water freezes, which most of us learn as children, happens at 0°C. But this is a truth with modifications; it is actually possible to have liquid water below 0°C [83]. If ice and water is mixed at 0°C, the two phases will exist in equilibrium, but if heat is removed the water will turn into ice. The water becomes solid while maintaining a temperature of 0°C. If only purified water is present, and no ice, the solidification follows a very different path because nucleation must precede crystal growth. Upon cooling, the temperature falls below the freezing point before nucleation and solidification. Liquid water below the freezing temperature is called supercooled water and it can, for example, be found in the atmosphere.

The first step in ice nucleation is the aggregation of water molecules into transient ice-like embryos, seed crystals [84, 85]. The embryos are not stable and can dissipate into the solution again due to thermal motion. Continuous fluctuations in the sizes due to the incorporation of new molecules and the detachment of others may result in an embryo growing large enough to become stable. There is a lower

limit on the size of an ice crystal, because it takes energy to create a surface. A minuscule ice crystal has a large surface-to-volume ratio and the free energy required to create the solid-liquid interface is larger than the gain in free energy from the change of metastable liquid (the supercooled state of water) into ice. The chance of an embryo reaching critical size increases with decreasing temperature and the duration of the low temperature. Eventually, an ice embryo achieves sufficient size to be stable, thus a nucleus is formed that becomes the basis for further growth. This process, involving only the spontaneous aggregation of water molecules, is called homogenous nucleation. This is a rare event in nature, and probably only takes place in the upper atmosphere where snow, rain and hail are formed. Much more common is the process where nucleation is catalyzed by pre-existing structures, such as mineral particles, organic compounds or surfaces, and this is called heterogeneous nucleation, see figure 14. Attachment of an embryo to a foreign surface can enhance the probability that a nucleus of critical size can form, by replacing part of the embryo-water interface with an embryo-surface interface and increasing the volume-to-surface ratio. Supercooled water is not at equilibrium and if an ice crystal or impurities are added, it freezes. In laboratory experiments, purified water has been found to freeze approximately at -40°C [83, 86].

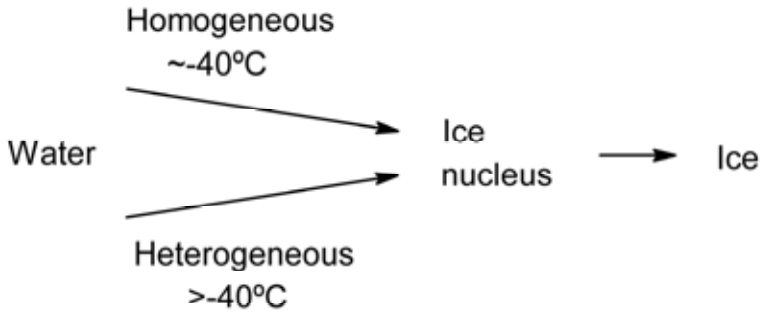

Fig. 14. The two paths to ice formation. Heterogeneous nucleation takes place when pre-existing structures catalyze the formation of ice nuclei and thus the freezing of water into ice. Homogeneous nucleation is the spontaneous aggregation of water molecules.

The freezing point of an aqueous solution is a colligative property, meaning that the freezing and melting temperature is related to the quantity of added solutes, but not to what type of solute. For example, glycerol or salts can lower the freezing temperature of water, but they also lower the melting temperature by the same amount and the particles do not interact specifically with the ice crystals [87]. Using a higher concentration of antifreeze in the car radiator gives a larger freezing point depression, thus giving protection at lower temperatures. For example, 25 % (by volume) ethylene glycol is enough, if the temperature only drops to ~-10°C, while at -20°C it is necessary to have 35 % glycol [88].

Properties of AF(G)P

In nature there exists a class of proteins that accumulate at the surface of seed ice crystals and inhibits ice crystal growth, thus lowering the freezing temperature specifically [89]. In the 1960s the investigations started to find the answer to why fish in the polar oceans and the near shore waters of the north temperate oceans do not freeze during winter [90-92]. The body fluids in teleost fish contain solutes that depress the fish freezing point to temperatures between -0.6°C and -0.8°C. This is enough for fresh water which

does not go below 0°C. Seawater, on the other hand, has a high salt concentration and freezes at -1.7°C to -2°C. Thus, when the temperature in the ocean falls below ~-0.7°C the teleost fish should freeze to death because they have a lower solute concentration than the surrounding icy seawater. However, freezing in these fish does not occur unless they are exposed to ice at temperatures below -2.2°C [93]. In contrast, temperate and tropical fish freeze at approximately -0.7°C and dies. The additional freezing point depression observed in polar ocean fish is caused by proteins or glycoproteins, which collectively are called antifreeze (glyco)proteins, AF(G)Ps. They act in a non-colligative manner and on a molar basis they depress the freezing point several hundred times more effectively than expected from their numbers only [94-96]. The freezing point depression is dependent upon concentration, but not linearly; there is a gradual leveling off at higher concentrations [96, 97]. The AF(G)Ps only have a colligative effect on the melting temperature, resulting in a difference between the temperature at which a seed crystal will grow in the solution and the melting temperature. This difference is called thermal hysteresis and is used as a measure of the non-colligative activity of the AF(G)Ps [97].

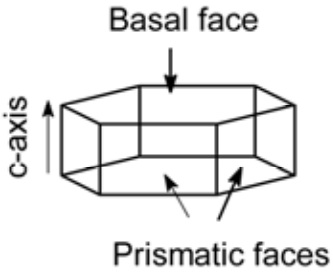

Fig. 15. Everyday snow and ice is hexagonal ice. A hexagonal crystal can be described as a prism with a hexagonal base.

Other phenomena associated with the AF(G)P is accumulation of protein at specific faces of the ice crystal, modification of the crystal morphology and inhibition of the growth of large crystals (recrystallization inhibition) [89, 98, 99]. Ice

crystals grow so that the surface with lowest energy has the largest surface area. In aqueous solutions under atmospheric pressure the basal face, see figure 15, usually has the lowest energy, hence the growth is much higher on the prismatic faces, resulting in flat circular discs [100, 101]. When single ice crystals are formed in AF(G)P solutions, within the thermal hysteresis gap, the crystal growth is arrested and the crystal morphology is altered, for example into a hexagonal bipyramid, see figure 16 [90, 97, 102-106]. Lowering the temperature below the hysteresis gap makes the crystals grow and the solution freezes to ice, but with an increased number of smaller ice crystals.

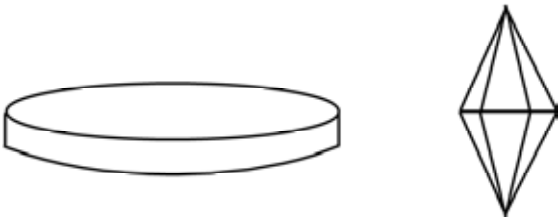

Fig. 16. Ice crystal shape when grown in aqueous solution to the left (flat disc) and in AF(G)P solution to the right (hexagonal bipyramidal).

According to the widely accepted adsorption-inhibition model the AF(G)Ps bind to the surface of specific planes of the ice crystals and thereby prevent further growth, but the exact mechanism by which this is achieved is not understood [90, 93, 97, 107]. It is hypothesized that the ice surface growing out between two adsorbed AF(G)P molecules will have a high curvature, and therefore high surface free energy, see figure 17. The surface-to-volume ratio will reach a limit where further ice growth is energetically unfavorable unless the temperature is lowered. This local depression of the freezing point is sometimes called the Kelvin or Gibbs-Thomson effect [97, 108-112]. Results from several studies have also indicated that the AF(G)Ps function independently of each other and bind to specific planes of the ice surface, but validation of the theory has yet to come [90, 91, 103, 110, 112-120].

Fig. 17. Schematic illustration of the Kelvin effect. The AF(G)Ps (black dots) adsorbed on the ice surface forces the ice to grow with a higher curvature between the proteins, than if the proteins were not present on the ice surface.

AF(G)Ps have now been found in fish [90, 93, 121-128], bacteria [129, 130], fungi [104, 129, 130], plants [99, 129-134], and invertebrates (insects, spiders, sponges, etc) [105, 106, 122, 135, 136] that experience freezing conditions in nature. The study of the AF(G)P-ice interaction is difficult and, unlike most protein-ligand interactions, the direct study of the protein-ice complex is almost impossible. The common functionality was believed to suggest shared structural elements or properties among the AF(G)Ps, but so far none has been identified. It has been found that it is actually a structurally diverse group of proteins that has evolved from different origins. The AF(G)Ps found in fish are the most studied and they can be divided into five types: antifreeze protein (AFP) type I-IV and antifreeze glycoprotein (AFGP) [90, 126-128, 137]. The thermal hysteresis activity of fish AF(G)Ps is typically ~1-1.5°C. Two of the types found in fish, AFP I and AFGP, were selected to be mimicked in this thesis.

The type I fish AFPs are small, alanine-rich (> 60%), α-helical proteins and are found in right-eye flounders and short-horn sculpins [95, 112, 138, 139]. They are the smallest and probably the simplest of the AF(G)Ps, as they consist of a single, long α-helix and therefore lack tertiary structure. The most extensively studied has been isolated from the winter flounder and has 37 amino acid residues forming a single α-helix with an internal salt bridge and terminal cap structures [95, 140-143]. It contains three repetitions of an 11-amino acid sequence which commence with threonine and displays repeated polar residues on one side of the helix. It was first assumed that hydrogen bonds

between hydrophilic side chains and the ice lattice was the dominant mechanism behind the ice-binding ability, but later studies have diminished the role of the hydrophilic side chains in ice-binding and suggested contributions from van der Waals interactions and entropy gains [90, 97, 122, 141, 144-153]. The currently proposed ice-binding face displays a mixture of hydrophilic and hydrophobic functional groups and encompasses the face of the helix which has threonine residues (four in total) interspersed with three alanine residues [97, 122, 151-157].

AFGPs can be found in Antarctic notothenioids and northern cods and were the first to be discovered of the AF(G)Ps. [92, 158-161]. They have a repeating sequence of three amino acids, (alanine-alanine- threonine)$_n$, in which the hydroxyl group of the threonine residue is glycosylated with the disaccharide β–D–galactosyl-(1→3)-α-*N*-acetyl- D-galactosamine, see figure 18 [162, 163]. Characterizing the AFGPs has been difficult as the isolation and purification is a laborious and costly process often resulting in mixtures [158]. Preliminary structure-function studies have been conducted, but little insights into ice-binding affinity and specificity at the molecular level has resulted. Chemical synthesis of AFGPs might seem to be an attractive alternative but the preparation of such systems is still a formidable synthetic challenge; a few synthetic AFGP have, however, been reported [158, 161, 164-166]. The saccharide moiety has been shown to be critical for ice-binding, since removal of the disaccharide leads to complete loss of antifreeze activity [158, 167, 168]. Studies have also indicated that the configuration of the sugar hydroxyl groups is important and that hydrophobic groups, like the acetamide (-NHCOCH$_3$) group and the γ-methyl group of the threonine residues, are key functional groups in antifreeze activity also for AFGPs [158, 167]. While other biological antifreezes tend to possess very rigid solution structures, the AFGPs seem to be very flexible, and studies have revealed no permanent secondary structures [158, 169, 170]. The AFGPs show a variation in molecular mass and some minor differences in the amino-acid composition. Proteins with larger molecular mass have been found to have higher hysteresis values than smaller.

The high molecular weight AFGPs have higher activities than AFP I, but when comparing the type I AFP with AFGPs of similar size, the AFP I has higher activity.

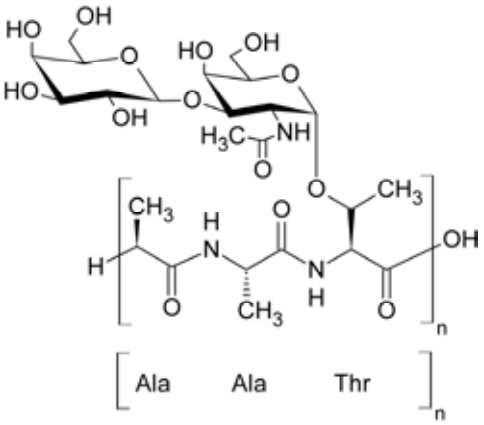

Fig. 18. Chemical structure of a typical antifreeze glycoprotein, n =4-50.

Mimicking AF(G)P

Preventing or modifying ice growth is related to the broader subject of biomineralization. The understanding of and ability to control crystal nucleation is still rudimentary, even though the process is of central importance to living organisms and to the fundamental and applied sciences. Insights gained from the study of AF(G)Ps can be applied to more commonly used minerals, for example, to alter calcite ($CaCO_3$) crystal morphology [171]. The design of a calcite binding peptide was based on the structure of an AFP I, where some of the ice-binding residues were replaced with aspartic acid to provide carboxyl groups [171]. The secondary structure of the protein is important in the presentation of binding functionality and thus to the morphology of the inorganic crystal [12]. Calcite is a common biomineral that can be easily grown as rhombohedra in the laboratory and the peptide was intended to bind to the prism faces producing hexagonal prisms with rhombohedral caps. When seed crystals and the peptide were added to the solution and growth was allowed to continue, a marked change in

morphology was observed. The crystals were elongated along the *c*-axis with rhombohedral caps, but no well-defined prism faces were seen. This effect was found to be critically dependent on the peptide structure; at conditions where the peptide lost most of the secondary structure, only 40% helix compared to 89%, this morphology was not seen.

Ice crystallization at a surface

There are still questions regarding the mechanism behind the thermal hysteresis seen for the AF(G)Ps, even after several decades of research, and this is also true for freezing at macroscopic surfaces. Extensive studies have been performed on water and ice at surfaces and in some areas, progress has been made, for example in understanding water behavior on most noble metals, but there are still many unsolved questions [84, 172, 173]. The possibility to influence the freezing temperature of water on a surface could be of use in many applications. For example, before take-off, ice is removed from airplanes with deicing fluid and then anti-icing fluid is applied to prevent accumulation of ice. At present large amounts of chemicals are used to deice airplanes and often the time the plane needs to be on the ground is increased. Other areas of which could benefit from this is power lines, bridges, refrigeration and several winter sports.

Some startling results have been obtained by studying ice nucleation in water drops covered by Langmuir monolayers of aliphatic long-chained molecules, see figure 19 [84, 174-179]. Freezing was observed at higher temperatures for alcohols, $C_nH_{2n+1}OH$, with longer chains and there were differences between odd and even n. In comparison, for carboxyl acid, $C_{n-1}H_{2n-1}CO_2H$, the nucleation temperatures were scattered in the range -12°C to -18°C for different n. For mixed Langmuir monolayers of $C_{31}H_{63}OH$ and $C_{32}H_{66}$, the ice nucleation temperature was found to decrease with decreasing alcohol content in the film, from -4°C for a 100% hydroxyl-terminated down to -15°C for a 100% methyl-terminated Langmuir monolayer [180]. Conclusions that were drawn were that nucleation activity is dependent on a good geometric match and increased order in the monolayer increases the nucleation temperature.

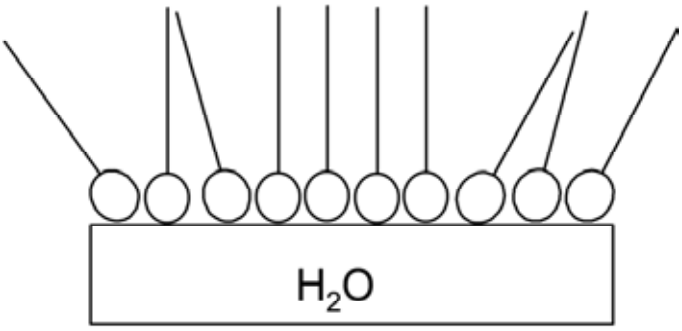

Fig. 19. Schematic illustration of a section of an uncompressed Langmuir monolayer of molecules with hydrophobic tails and hydrophilic heads.

Model surfaces

In this thesis two attempts to mimic the active domains of AF(G)Ps at a macroscopic surface is described. One model system was based on the AFGPs and consisted of mixed SAMs of an alanine analogue and a disaccharide analogue. The monolayers showed well-ordered alkyl chains up to a disaccharide surface fraction of 0.8, while monolayers of higher disaccharide fractions showed an increase in *gauche* defects and were less densely packed. Water was condensed on the SAMs and the crystallization to ice was studied via an optical microscope. Differences were observed compared to a reference system composed of mixed SAMs of hydroxyl- and methyl-terminated alkanethiols. Overall freezing was observed from -5°C to -14°C, with a general trend of decrease in temperature when the hydrophobicity increased, or alcohol content decreased, which is in good agreement with the studies of ice nucleation temperatures for mixed Langmuir monolayers mentioned above. For SAMs with a higher surface fraction of disaccharide than ~0.3 a plateau at about -5°C was observed. For disaccharide fractions similar to the active domain of AFGPs the crystallization temperature was higher than reference monolayers with comparable contact angles.

The other system was based on AFP I and consisted of mixed monolayers of an alanine analogue and a threonine analogue. Well-ordered monolayers were observed for all surface fractions of alanine, 0-100%, in this system. The study of the crystallization of water did not reveal any differences compared to the reference system, the same -OH/-CH_3 system as the one used in the study of the AFGP model. Freezing was observed in the range -10°C to -14°C, and an increase in hydrophobicity, or decrease in alcohol content, decreased the crystallization temperature. Deposition in ultra high vacuum of D_2O ice also showed behavior in good agreement with earlier studies of water on mixed SAMs of hydroxyl- and methyl-terminated thiols.

Experimental techniques

This section describes the main experimental techniques used in this thesis to make the models and to characterize them.

Substrate preparation

Flat, centimeter-sized gold surfaces were prepared from silicon wafers to be used as substrates for SAM formation in this thesis. The silicon (100) wafers were cut in desired sizes, cleaned and then polycrystalline gold films, 2000 Å thick, were deposited with a thin layer, 25 Å, of titanium in between to increase the adherence of gold to the silicon. The metals were deposited on the slides in ultra high vacuum, 10^{-9} - 10^{-10} mbar in base pressure. The metal was heated, and thus evaporated, by an electron beam and the metal vapor adhered to the slides. The surfaces prepared in such a setup have been shown to have a (111) grain size of 200-500 Å and the outermost surface of the grains has a terrace like structure with large flat regions [56, 181, 182]. It is very important to remove impurities on the gold surfaces before the formation of high quality SAMs and therefore they were chemically cleaned before use. This treatment also reduces the surface roughness and increases the size of the grains to ~1000 Å [183].

Self-assembled monolayer

Self-assembled monolayers (SAMs) have become an important research technique to produce surfaces with very well defined chemical composition. SAMs make it possible to tailor and optimize the properties of a surface for fundamental studies of surface phenomena and for use in applications. They are used in applications for molecular recognition, as protective coatings, to control the friction of a surface, as attachment for further layers, and in the study of nucleation, protein adsorption, corrosion, cell adhesion, wetting, etc [184-191].

A SAM is a two-dimensional film, one molecule thick, spontaneously assembled by chemisorption at a surface, not necessarily with planar geometry [192]. The thickness of these monolayers is typically in the nanometer range in the vertical direction and, as a result, they can be called nanoassemblies. In the horizontal plane, i.e. over the substrate surface, these monolayers can, however, extend on the macroscopic length scale. Several systems of molecules on surfaces are included in this concept. The requirement is that they contain a chemical group (the head group) that can form a bond to the surface, in most cases through chemisorption. A wide range of adsorbates have been studied, for example organosilicon on hydroxylated surfaces, carboxylic acids on metal oxides and organosulfur on semiconductor, nickel, iron, copper, platinum, silver and gold surfaces [181, 189-191].

The models in this thesis are based on SAMs formed of alkanethiols on gold substrates [78, 181, 185, 189, 190, 193, 194]. Thiols are similar to alcohols (R-OH) but with the oxygen replaced with a sulfur atom (R-SH). The alkanethiols have three parts, head group, tail group and an alkyl chain, see figure 20.

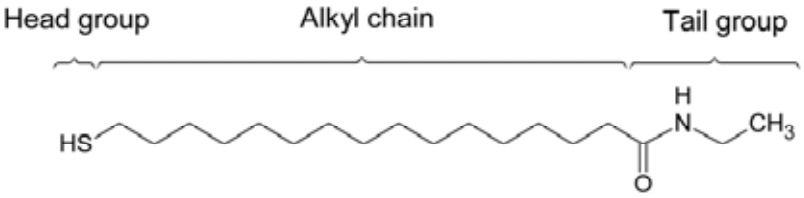

Fig. 20. Thiol analogue of alanine in an extended, all-*trans* (zigzag) conformation.

The sulfur atom in the head group (-SH) forms a strong and specific interaction with the gold surface [195-197]. A very high chemical variability is possible in the tail group and the alkyl chain can also be exchanged to other structures like aromatic rings. Gold is relatively inert, allowing for adsorption of organosulfur compounds exclusively through the sulfur atom(s), and it does not form an oxide when exposed to air in the laboratory, thus letting the substrate be manipulated without overly caution [181, 195]. SAMs on gold is a widely used molecular film system since it is easy to prepare and offers a large variability. It also has the advantage that it can be efficiently characterized using optical, mechanical and electrochemical methods.

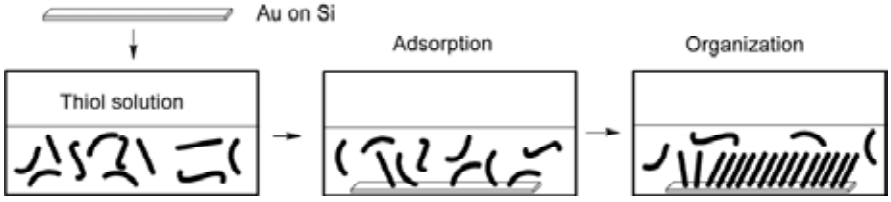

Fig. 21. Schematic illustration of the preparation of a SAM. The substrate is immersed in a solution of the desired thiol(s). Initial adsorption is fast, seconds, and then follows an organization phase, hours, where the order increases.

The monolayer forms spontaneously when a clean gold surface is submerged in a thiol solution, see figure 21. The

molecules immediately attach to many binding sites on the surface, but initially they are disordered and may sterically block some sites. If the incubation continues the thiols will rearrange from an initial distribution of conformers into a more ordered monolayer and this may take hours or days [190, 195, 198]. The incubation time can thus vary from a few minutes up to several days, depending on the type of alkanethiol, the concentration, and how highly ordered the monolayer is desired to be.

The mechanism of thiol chemisorption to gold is not completely understood, but the theory is that the thiol, $X(CH_2)_nSH$, is deprotonated and bound on the surface as a thiolate, $X(CH_2)_nS^-$, where X is the tail group [185, 199-201].

$$X(CH_2)_nSH + Au \rightarrow X(CH_2)_nS - Au + \frac{1}{2}H_2$$

The distance between adjacent sulfur atoms of the monolayer is ~ 5 Å, and the S-S interactions are believed to be at a minimum [78, 192, 197, 199, 202, 203]. The distance between the chemisorbed sulfur atoms is also greater than the distance, ~4.6 Å, between closely packed alkyl chains [191, 196]. To maximize the interchain van der Waals interactions, the hydrocarbon chains of a highly ordered monolayer of alkanethiols adopt a largely *trans* conformation and are tilted 25-35° from the normal to the surface, see figure 22 [78, 181, 182, 184, 203, 204]. The S-Au interaction together with the van der Waals interactions between the chains are responsible for overcoming the decrease in entropy during the formation of well-ordered SAMs.

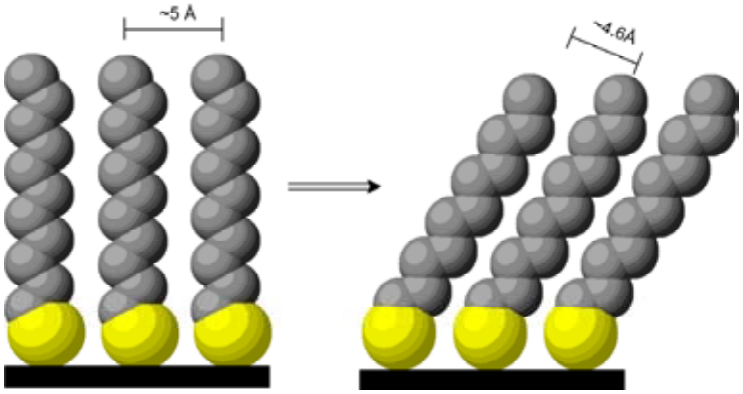

Fig. 22. Schematic illustration of the interchain distances for different packing in the SAM, with the tilted and more favorable packing to the right.

The van der Waals interactions between adjacent alkyl chains increase with the length of the chain and if the alkyl chain is longer than ten methylene groups, n > 10 $(CH_2)_n$, it is more favorable to form monolayers with the chains predominantly in an all-*trans* (fully extended, minimal disorder) conformation [78, 182]. Another factor influencing the packing of the chains is the size of the tail group; if it is larger than the alkyl chain it can interfere with the packing of the chains. In this thesis, this was observed for SAMs with a high fraction of the bulky disaccharide analogues. Electrostatic interactions, such as hydrogen bonds between the thiols, can also impart stability to the SAM. The amide group (-CONH-) present in the thiols used in this thesis, has previously been showed to increase the interchain interactions by forming hydrogen bonds [191, 205-207].

Monolayers formed from thiol mixtures add to the flexibility in design and synthesis of functional SAMs [56, 208-210]. The interfacial properties can then be continuously tuned simply by varying the ratio of the thiols in the mixed SAM. In this work monolayers, formed from both single and mixed thiol solutions, were studied. The alanine analogue was for example mixed together with the threonine analogue to form a biomimetic surface of type I AFP. The relative proportion

of the two thiols in the SAM depends on several parameters like the mixing ratio in the incubation solution and the solubility of the thiols in the solvent [188, 208-211]. In the case of the alanine and threonine analogues, the composition of the SAM was almost identical to the composition of the solution, which was expected since they are similar in chain length, solubility, and bulkiness of the tail group. However, the two thiols used to form a biomimetic surface of an AFGP are more different, with one glycosylated analogue, and thus the composition of the SAMs differed from the composition of the incubation solutions.

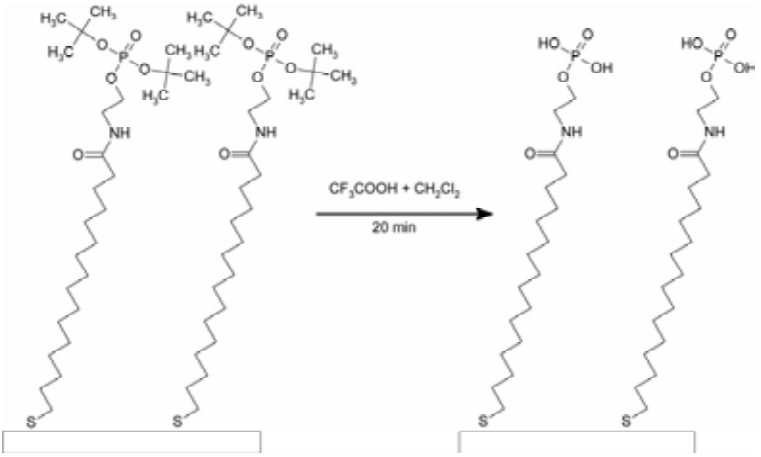

Fig. 23. Removal of the protective groups from the long-chained phosphorylated serine analogues in a SAM.

Chemical modification of the tail group of a SAM after assembly can expand the range of functionalities and molecules like biotin and proteins can be attached [56, 185]. The SAM system also provides controllable and well-defined models for fundamental studies of the influence of a surface on chemical reactions of functional groups. In this thesis, the long-chained phosphorylated amino acid analogues were modified with protective groups to make them possible to work with and to get well-ordered monolayers. The protective groups were then removed with acid after the monolayers were formed, see figure 23.

Ellipsometry

A newly made monolayer was usually first characterized by measuring the film thickness, and one simple and fast technique for this is ellipsometry [78, 212, 213]. The electric field of polarized light can be described by two orthogonal cosine functions, traveling in the direction of the light [212]. If these have a phase difference of $\pm n\pi$, where n is an integer, the light is linearly polarized. If the phase difference instead is $\pm\pi/2+2n\pi$ and the amplitudes of the components are equal, the light is circularly polarized. Both linearly and polarized light are special cases of elliptically polarized light, where the electric field vector both rotates and changes magnitude, see figure 24. Linearly polarized light reflected from a surface changes into elliptically polarized and this change in phase and amplitude is analyzed in ellipsometry. From the measurements it is possible to extract information like the thickness of a film on the surface, refractive index, surface roughness and anisotropy through a model-based analysis using optical physics [78, 210-212, 214].

Fig. 24. The electric field vector for linear (left), circular (middle) and elliptically (right) polarized light with the light propagating out of the page towards the reader.

For the measurements in this thesis a null ellipsometer was used, see figure 25 [212]. Monochromatic light (He-Ne laser) is plane polarized by a polarizer and a compensator changes the phase difference. The now elliptically polarized light is then reflected on the sample, is passed through a second polarizer, called analyzer, and then the light intensity is detected. This configuration operates by adjusting the orientation of the polarizer and analyzer so that the reflected light is extinguished or “nulled” and no light is incident on the

detector. This is possible when the polarizer is at such an angle that the light reflected from the surface becomes linearly polarized, which can then be extinguished by the analyzer.

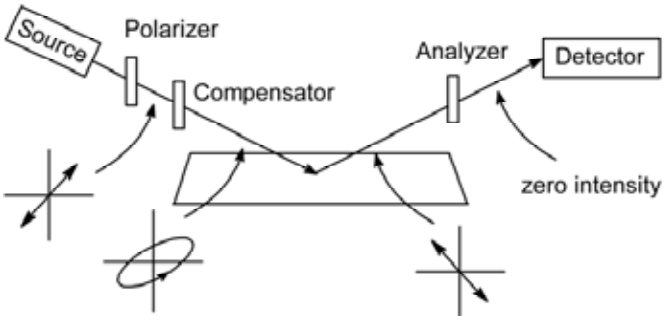

Fig. 25. The principle of the polarizer compensator sample analyzer (PCSA) null ellipsometer.

In a two-phase system, i.e. a pure gold surface in air, it is straightforward to calculate the complex refractive index for the substrate, the gold surface, from the two measured angles from the polarizer and the analyzer. In a three phase system, like a SAM on a gold surface in air, it gets more complicated; the refractive indices for the gold and the SAM have to be known to get the film thickness. For SAMs of alkanethiols a refractive index of $1.5 + 0i$ has been shown to yield accurate results [78, 181, 215]. The gold refractive index was easily obtained since the gold surfaces were measured before forming the monolayer to check the quality of the gold films; thus giving the gold refractive index. With the refractive indices known it was possible to determine the thickness of the SAM with ellipsometry [214].

Contact angle goniometry

Information about surface energy, surface roughness and surface heterogeneity can be obtained with contact angle goniometry [181, 216, 217]. This method studies the surface wettability, i.e. what happens when a liquid is brought into contact with a solid surface by measuring the contact angle between the liquid-gas and solid-liquid interfaces. If the

substrate has an attractive force on the liquid, the surface is called lyophilic and the liquid will have a relatively large contact area with the surface, wetting the surface. If the force is repulsive, the surface is lyophobic and this is called dewetting. The most known example of this is where the liquid is water and in that case the mentioned surfaces would be hydrophilic and hydrophobic, respectively.

A liquid drop on a substrate is bound by two types of interfaces, the liquid-vapor interface and the liquid-substrate interface. These two surfaces meet along the edge of the liquid surface, if the liquid does not wet the surface completely. The angle at which these two interfaces intersect is known as the contact angle, θ, see figure 26. The contact angle is related to the surface tension γ of the interfaces as given by Young's equation:

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos\theta$$

where the indexes s, l and v mean solid, liquid and vapor [217, 218]. Thus, γ_{sv} is the surface tension between solid and vapor.

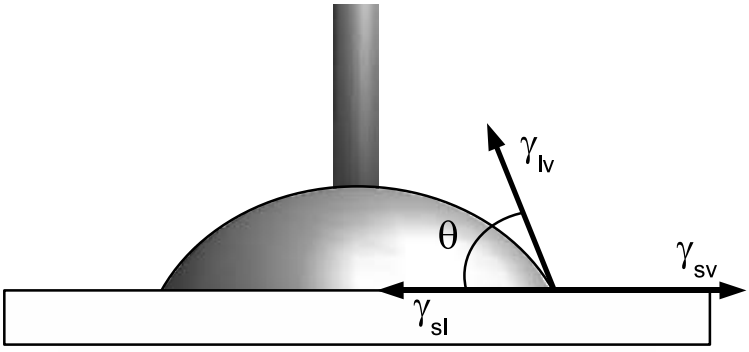

Fig. 26. Contact angle setup with the needle inserted into the liquid drop on a solid surface to be able to measure advancing and receding angles. Surface tensions (γ) and contact angle (θ) is marked in the figure.

This technique does, however, have a strong empirical character and there is some controversy regarding the extraction

of information from the measurements [216, 217, 219]. Real surfaces, for example, do not exhibit a true and unique contact angle; they usually display a range of allowed contact angles [23]. The advancing angle, θ_a, (the maximum angle) is determined with the help of a contact angle goniometer by slowly adding liquid to the drop. This is done with a syringe mounted above the drop with the syringe needle in contact with the liquid, see figure 26. The receding angle, θ_r, (the minimum angle) is then determined by removing liquid from the drop. The difference between these two angles is called hysteresis and depends on surface heterogeneity and roughness [23, 181, 195, 217, 220].

Thiol analogue	Alanine	Serine
Advancing water contact angle	97°	28°

Different tail groups in a SAM give different contact angles. Changing the methyl in the alanine analogue to a hydroxyl in the serine analogue changes the advancing water contact angle from 97° to 28°. Thus, the contact angle measurement is a useful technique in the study of SAMs [193, 195, 200, 208-211, 213, 220, 221]. In this thesis the contact angle analysis of SAMs did not include calculations of the energetic parameters of the surface, but rather gave a general indication about the exposure of the terminal groups and as a comparative basis. This was done with two liquids, water and hexadecane [195, 208-211, 213, 220]. Water probes the polar characteristics of the surface and hexadecane probes the apolar or dispersive characteristics.

Contact angles can also be used to get an estimate of the composition of a mixed SAM by a relation suggested by Cassie:

$$\cos\theta = \sum_i \chi_i \cos\theta_i$$

where χ_i is the fraction of the i:th component and θ_i is the contact angle in a homogeneous SAM of i [193, 219, 222]. In this thesis this was used to estimate the fraction of disaccharide analogue at the surface for the SAM model of AFGP.

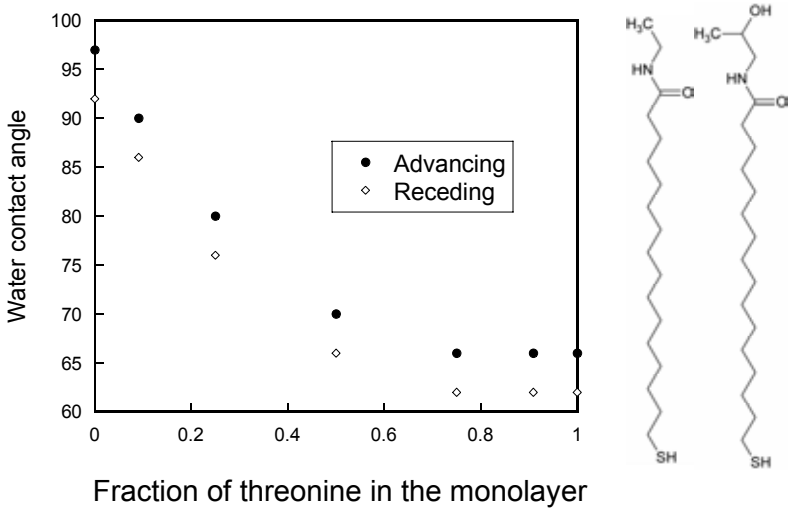

Fig. 27. Contact angles on AFP I model surfaces formed from a mixture of alanine (left) and threonine (right) analogues.

The hysteresis is also believed to give some indication of the distribution of thiols in mixed monolayers [208]. For example, if the threonine and alanine analogues formed macroscopic segregated domains in the mixed monolayers of the AFP I model, the nonpolar (alanine) domains would hinder the advancing water drop while the polar (threonine) domains would pin the receding water drop. Consequently, the hysteresis would be much greater on the mixed monolayers than on a SAM of only threonine or alanine analogue. This dependence on polarity was not observed; in fact the hysteresis was slightly larger on the alanine monolayer, see figure 27.

Spectroscopy

When an electromagnetic beam is passed through a sample it can be absorbed or transmitted. What happens is dependent upon the frequency of the light and what the sample contains. If a molecule absorbs light, it gains energy and undergoes a transition from one energy state to another. If infrared light is used, 4000 – 400 cm^{-1} or 2-20 μm, there is

a transition from one vibrational energy level to another and the technique is known as infrared spectroscopy [223]. Infrared spectroscopy is widely employed for the recognition and the quantitative analysis of structural units in a sample. It provides essential information in terms of the constituent groups and their intra- and intermolecular interactions. If light of higher energy is used, for example X-rays, the transition changes the electronic configuration of the atoms by having enough energy to eject electrons from the atom. X-ray photoelectron spectroscopy (XPS) studies electrons that have escaped from the inner electron shells [224]. XPS is commonly used to determine the elemental composition of a sample. It can also give some information about intramolecular interactions and the distribution of the elements in the sample.

Infrared spectroscopy

Two covalently bound atoms are held together by an electron cloud and this, in a very simple model, may be thought of as a spring connecting two spheres. When the spheres are set in motion, they will vibrate back and forth on the spring, or oscillate, at a certain frequency depending on the masses and the stiffness of the spring. Smaller masses oscillate at a higher frequency because they are lighter and easier to move than larger masses. A stiffer spring, or bond, will also oscillate at a higher frequency because it is harder to deform and quickly returns to its original state compared to a weak spring. Other vibrations in infrared spectroscopy come from deformations of a group, i.e. changes of the angle between bonds. The deformation vibrations can be found at lower frequencies than corresponding stretching frequencies; it is easier to bend a bond than to stretch or compress it. In many modes only a few atoms are moving, for example a functional group, while the rest of the molecule is almost stationary. The frequencies of such modes are then characteristic of the specific functional group which is in motion, and are only minimally affected by the nature of the other atoms in the molecule. However, intermolecular interactions involving the group, for example hydrogen bonding, will influence the frequency of the vibration.

Transmission spectra showing what frequencies that are absorbed when the infrared light is passed through the sample can be obtained from samples in all phases, gas, liquid and solid. For example, if the sample is a powder it can be finely grounded together with KBr, a salt that absorbs very little in the infrared region, and then pressed into a thin disk before measurement. This method was used to collect bulk spectra of all thiols in solid form in this thesis, while the liquid thiols were smeared on windows of salt. This means that the molecules were randomly oriented in the bulk samples. A reference spectrum, for example a disk of only KBr, was used to remove contributions in the sample spectrum from the mirrors, beamsplitter, windows, light source and detectors.

A special setup called infrared reflection-absorption spectroscopy, IRAS, is needed to study monolayers on metal surfaces [223, 225, 226]. In IRAS, the infrared light hits the thin film at a near-grazing angle, passes through the film, and is then reflected by the highly reflecting surface of a metal, passes through the thin film again and then hits the detector.

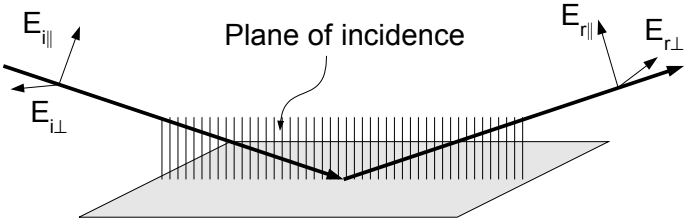

Fig. 28. Schematic illustration of the light in the IRAS setup. The plane of incidence is defined as that plane which contains the incident light, the reflected light and the surface normal (the vector which is perpendicular to the surface).

The electric field of the incident and reflected light can be separated into two components that oscillate parallelly, $E_{\parallel}$, and perpendicularly, $E_{\perp}$, to the plane of incidence, see figure 28. The perpendicular component is almost entirely can-

celled in the thin film, $E_{r\perp} = -E_{i\perp}$, for all angles of incidence, due to a phase shift of nearly 180° when the light is reflected, and the interaction with adsorbed molecules is insignificant. The parallel components of the incident and reflected light do not cancel. At high angles of incidence they combine and form a strong electric field perpendicular to the surface, $E_{i\parallel} + E_{r\parallel} \approx 2E_{i\parallel}$. This means that only vibrations with a component of the transition dipole moment oriented perpendicular to the metal surface will cause absorption [181, 225, 226]. This is referred to as the "surface dipole selection rule". The spectra, therefore, provide information about the orientation as well as the composition of the sample films. This is true for thin films where the thickness of the film is small compared to the wavelength of the infrared light. In thicker films the light will travel through the film while it is unaffected by the reflection and thus the surface selection rule will not apply.

SAMs are mostly formed by highly organized and oriented molecules, and therefore, some of the peaks that are normally seen in an isotropic (with randomly oriented molecules) spectrum will appear with low or zero intensities in IRAS, due to the surface selection rule, see an example in figure 29. Other vibrations that are more favorably oriented will show increased relative intensities. This phenomenon can be used to determine the molecular orientation in thin films by comparing the IRAS intensity of the film with that of the transmission spectrum of the bulk isotropic sample, measured for example using a KBr pellet [182, 194, 206].

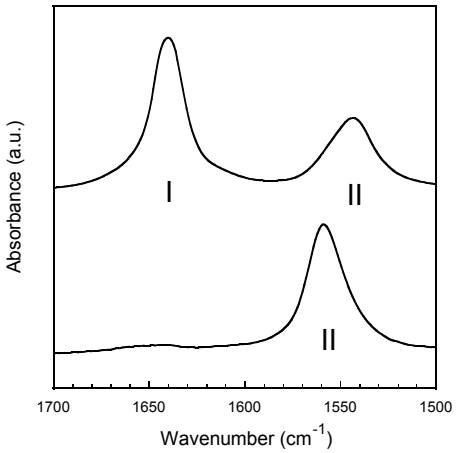

Fig. 29. Comparison of transmission spectrum of isotropic KBr sample (top) and monolayer IRAS spectrum (bottom) for the amide I and II band showing the effect of the surface selection rule for a highly oriented SAM. Amide I is oriented parallel to the surface in the SAM and thus not visible in the spectra.

The degree of order in the monolayer can also be measured with IRAS, for example by observing the CH stretching vibrations which are very sensitive to packing density and to *gauche* defects [78, 181, 182, 206, 207, 220, 227]. The alkyl tails vibrate at characteristic frequencies and both the width of these peaks and the frequencies of the vibrations give a picture of the relative order within the SAM. If the chains are disordered, the neighboring chains impose less constrains and allow a larger distribution of frequencies, thus increasing the width of the peaks. The position of the antisymmetric CH_2 stretching vibration, for example, varies from 2916-2917 cm^{-1}, for SAMs of exceptional quality, up to 2928 cm^{-1}, measured for alkyl chains in the liquid state, where the alkyl chains are heavily disordered [78, 227]. Many highly ordered SAMs display the antisymmetric CH_2 stretch at 2918-2920 cm^{-1} [182]. A parameter that influences the packing of a SAM is the length of the alkyl chain, $(CH_2)_n$; for short alkanethiols ($n \leq 9$) it appears that the monolayers are more liquid-like, whereas long alkanethiols

($n \geq 10$) are packed like crystalline polyethylene [78, 182]. Bulky tail groups, such as the disaccharide in this thesis, can also affect the packing of the chains. A SAM of only the disaccharide thiol is not crystalline-like, but if the chains are "diluted" with smaller thiols, as in this thesis with alanine analogues, it is possible to achieve highly ordered SAMs.

In this thesis, a monolayer of deuterated thiols was used as reference. Deuterium is heavier than hydrogen, and thus the peaks are shifted and do not interfere with the peaks from the sample.

X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical applications (ESCA), is a surface sensitive technique, which is suitable for characterizing SAMs [181, 195, 199, 200, 210, 213, 222, 224]. It is based on Einstein's photoelectric effect where the incident photon "disappears" and one electron is ejected from the absorbing atom, leaving the atom in an excited state. The kinetic energies of the photoemitted core-level electrons that leave the sample are measured and a spectrum is recorded. The kinetic energy of the photoelectrons is given by the difference between the photon energy and the atomic binding energy of the electrons. The binding energies can thus be calculated and used to determine the composition since they are characteristic for each element. XPS can also be used to determine the ratio of thiols in mixed monolayers [181, 200, 208-210]. To determine surface concentrations in mixed monolayers, it is important to study the end points, i.e. monolayers consisting of only one of the mixture components, and establish values for the "100%" surface concentrations. The fraction in the mixed monolayer can then be determined.

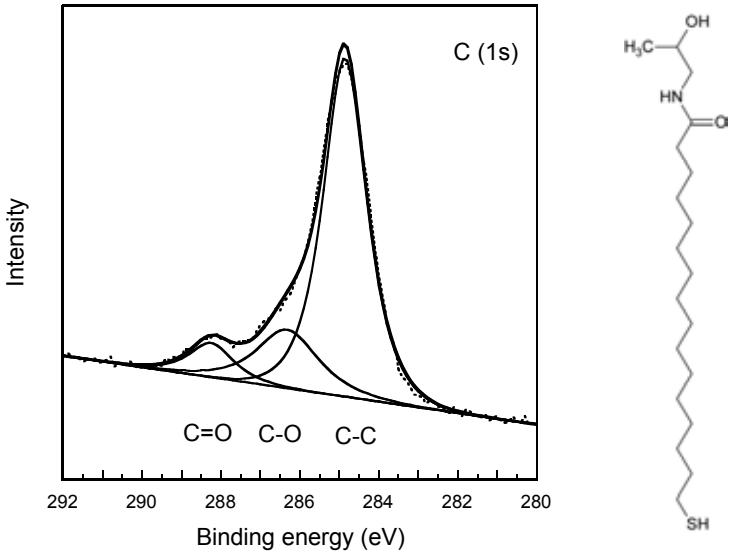

Fig. 30. C (1s) spectrum for a SAM of threonine analogue (right) showing three different environments for the carbon atoms.

Small variations in the binding energy, chemical shifts, can be seen since core electrons are affected by the valence electron distribution, see figure 30. Thus it is also possible to obtain some information about the chemical bonds between neighboring atoms.

XPS is a surface sensitive technique since only electrons emitted very close to the surface will contribute to the observed intensities in the spectrum [181, 224, 228]. The electrons can not move more than a few nanometers through the material before they get absorbed due to a high probability of inelastic scattering of the electrons inside the sample. This property can be utilized to determine the vertical structure of SAMs [195, 199, 229]. Because of the limited inelastic mean free path of the electrons, depth dependent information about the atoms in the near surface region can be obtained by varying the angle between the surface normal and the entrance to the electron analyzer. The path through the sample is considerably extended for electrons

originating from the part of the SAM closer to the Au surface when exiting in a direction nearly parallel to the surface; thus the probability of attenuation by the overlaying material is increased. In the surface sensitive mode the electron intensity from the outermost atoms is enhanced relative to that from the deeper laying atoms and the intensity of the peaks corresponding to gold and sulfur decreases. The combination of XPS and the "surface selection rule" in IRAS provides a powerful tool for studying the orientation of adsorbed molecules on metal surfaces.

Threonine analogue	O/S relative intensity ratio
Theoretical value for the thiol	2
Bulk mode for SAM	2.1
Surface mode for SAM	4.9

Summary of the papers

The common topics of paper I-V are: creating new organic interfaces and evaluating their characteristics. The papers are all based on a biomimetic approach to designing new materials. The interfaces were created by utilizing self-assembled monolayers (SAMs) of alkanethiols on gold substrates. Paper I-III concerns the area of phosphorylated surfaces and paper IV-V deals with mimicking antifreeze (glyco)proteins.

Properties of phosphorylated surface models, paper I-III

The work presented in paper I-III is an attempt to study and utilize the phosphate functionality. As mentioned earlier phosphorylated surfaces are of interest in both fundamental research and in possible applications in for example biomaterials and sensors. It is important to develop new and refined model systems (natural as well as artificial).

The model systems in these papers were created with SAMs of alkanethiol analogues of serine, threonine and tyrosine, with and without a phosphate group, formed on macroscopic surfaces and characterized by surface analytical techniques. With access to amino acid analogues with and without phosphate groups, surfaces with any desired mix-

ture of phosphorylated to non-phosphorylated amino acid analogues can be realized.

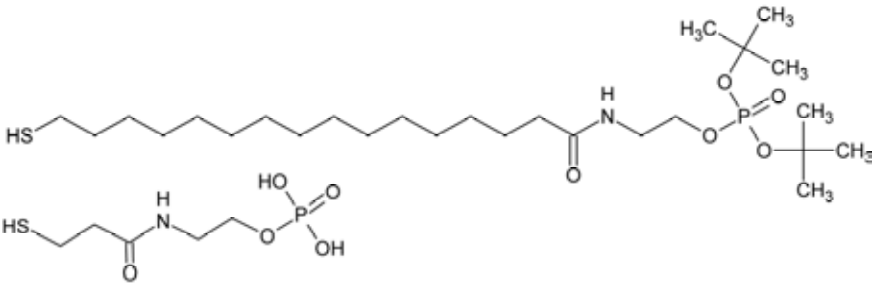

Fig. 31. Long- (top) and short-chained (bottom) phosphorylated serine analogues. The protective groups are removed from the long-chained analogues after assembly into a monolayer.

With the purpose of creating highly organized monolayers with good insulating properties the analogues were synthesized with long, $(CH_2)_{15}$, alkyl chains. Due to solubility and handling problems encountered during the work with long-chained phosphates, two strategies were tested to overcome these problems. The first was to mask the phosphate functionality on the alkanethiols with *tert*-butyl groups, see figure 31. The protective groups were removed after assembly into monolayers. The other strategy was to use a shorter alkyl chain, consisting of only two methyl groups. The phosphorylated analogues with a short chain can simply be assembled just as the non-phosphorylated analogues without the need of further reactions.

Results from paper I

In this paper were the phosphate model surfaces created with long-chained thiol analogues of the amino acids serine, threonine and tyrosine, see figure 32. All the SAMs were characterized using infrared spectroscopy, ellipsometry, XPS and contact angle goniometry. The results from ellipsometry and IRAS showed that the tyrosine analogue formed highly ordered monolayers with the alkyl chains in an extended all-*trans* conformation and displayed a low contact angle for water (12°), in good agreement with exposure

of the hydroxyl (-OH) tail group. The serine analogue, that also has a hydroxyl tail group, formed highly ordered SAMs but with a slightly higher contact angle (28°). The observed difference between the serine and tyrosine SAM could be a consequence of the structural variations near the hydroxyl group since the liquid is capable of probing the chemical features a few angstroms into the monolayer. The XPS measurements did, however, show that the serine SAMs seem to have a small fraction of unbound thiols at the surface, perhaps as a second overlayer that partly penetrates into the first chemisorbed monolayer, which would affect the contact angle. Unbound thiols were not observed for monolayers of tyrosine or threonine analogues. The threonine analogue formed highly ordered monolayers with an intermediate contact angle (66°), in good agreement with the mixed characteristics of its tail group, containing a hydroxyl and a methyl group (-CH_3).

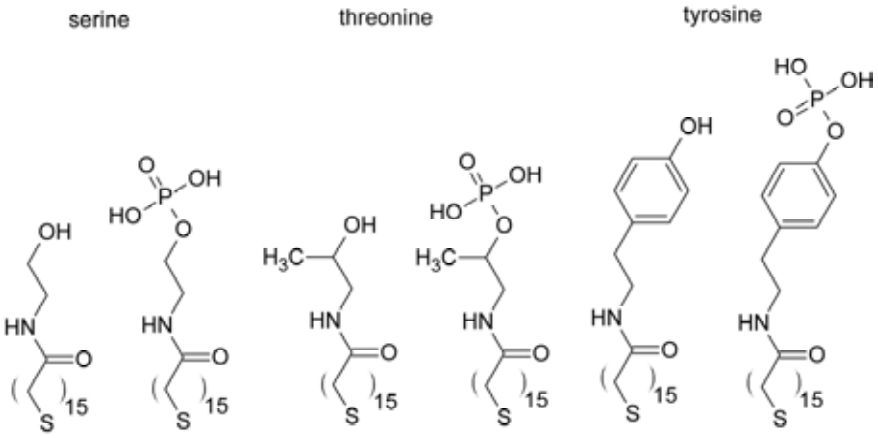

Fig. 32. Amino acid analogues of serine, threonine and tyrosine in the long-chained phosphate model SAMs.

The protected phosphorylated analogues formed well-ordered SAMs, but the amount of *gauche* defects in the alkyl chains were higher than for the non-phosphorylated analogues. The deprotection performed after assembly was successful in completely removing the *tert*-butyl groups, but

it did slightly increase the amount of *gauche* defects in the alkyl chains. The SAMs were, however, still well-ordered, for example, the asymmetric CH_2 stretching vibration was found at 2920 cm^{-1} for both the phosphorylated serine and tyrosine analogues. The phosphorylated monolayers displayed contact angles which were dependent on the underlying functional groups indicating that water can penetrate past the phosphate groups. The incubation time was proven to be a critical parameter in achieving high quality monolayers for the phosphorylated analogues. SAMs formed after 20 h of incubation showed a rather large fraction of wrongly incorporated thiols in the assembly, which were not present after only 12 h of incubation. This was evident in the XPS spectra where unbound thiols were present in the outer part of the SAMs for the longer incubation even after ultrasonication, but no unbound thiol was discernable for the shorter incubation time.

Thus, the thiols formed ordered SAMs exposing the tail groups as planned and the phosphate model surfaces were ready to be used (see paper II).

Results from paper II

Electrochemical measurements were performed on SAMs of the long-chained thiols to demonstrate the possibility of electrochemical detection of the phosphate group. Initially, the insulating properties of the monolayers were tested by cyclic voltammetry and were found to be sufficient for all analogues. This also showed that even though deprotection of the assembled phosphorylated thiols induced some *gauche* defects in the monolayer it did not significantly affect the insulating properties.

The change in surface capacitance upon phosphorylation of the alcohol group was then evaluated by impedance measurements on the different monolayers. The introduction of the protected phosphate groups changed the capacitance, but with a smaller change for the tyrosine analogues where the aromatic ring structure appeared to be “buffering” the polarity of the phosphate group. The removal of the *tert*-butyl groups introduced charges in the monolayers and the capacitance increased even more. The polarity and charge

introduced by the phosphates was found to contribute to an overall ~40% change in the capacitance. A difference was thus readily detectable between non-phosphorylated and phosphorylated monolayers. This indicated that processes, such as phosphorylation, that changes the polarity or charge of a monolayer on an electrode can be followed by evaluating the interfacial capacitance.

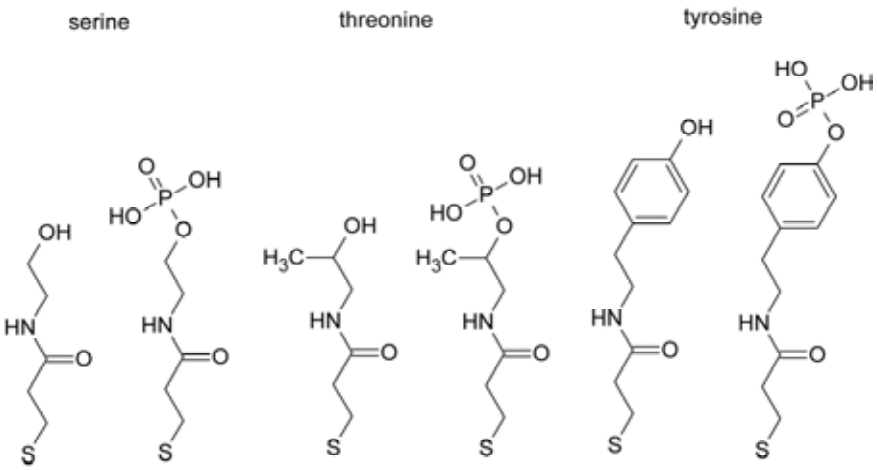

Fig. 33. Amino acid analogues of serine, threonine and tyrosine in the short-chained phosphate model SAMs.

Results from paper III

Monolayers of phosphorylated and non-phosphorylated amino acid analogues with a short alkyl chain were assembled and characterized using IRAS, XPS, ellipsometry, and contact angle goniometry, see figure 33. The amino acid analogues of serine, threonine and tyrosine formed monolayers with the expected thicknesses. IRAS showed that they were oriented with the C=O bond preferentially aligned parallel to the gold surface, just as for the long thiols. It was, however, not possible to discern anything about the configuration, *trans* or *gauche*, of the alkyl chain. The observed thicknesses for the phosphorylated monolayers were somewhat higher than expected, which could be

due to hydration of the phosphate or an erroneous ellipsometric model. The SAMs, except for those of the threonine analogue, showed high wettability as expected for SAMs of polar tail functionalities such as hydroxyl and phosphate groups.

The interactions of the phosphorylated serine analogue with the cations H^+, Ca^{2+}, Na^+ and Mg^{2+} were also studied. Counter ions were exchanged on the surfaces and XPS measurements showed only the presence of the intended cation on the SAMs. The ratio of counter ion versus phosphorous were close to one for all cations, excluding hydrogen, which is not possible to measure with XPS. Exchanging the counter ion did not affect the thickness or contact angle of the SAM. The infrared spectra of H^+-, Ca^{2+}-, Na^+-, and Mg^{2+}-treated SAMs, differed from SAMs measured directly after assembly, and demonstrated the importance of the phosphate counter ion. The largest effect upon ion exchange was observed in the outermost part of the molecules, which is seen as shifts in the peak of PO_3 stretching vibration. The singly charged ions, H^+ and Na^+ were loosely coordinated while the doubly charged ions, Ca^{2+} and Mg^{2+}, were more strongly coordinated. Of calcium and magnesium, is magnesium usually the more strongly phosphate-coordinating ion. This was confirmed with IRAS, where a slightly higher wavenumber of the phosphate stretch vibration for the magnesium-treated SAM indicated that this ion was the more strongly bound. Change of coordination is known to cause phospholipids to pair up and form clusters and this could indicate that the surfaces could be useful for studying interactions with phospholipids and phospholipid bilayers. The phosphate structure, seen in infrared, upon ion exchange with magnesium and calcium was also found to be similar to the structure of hydroxyapatite. This similarity is important for initializing the crystallization of inorganic salts and will hopefully enable the utilization of the surfaces as templates for biomineralization.

Properties of antifreeze (glyco)protein models, paper IV-V

Ice on surfaces causes many problems and attempts have been made to reduce the freezing of ice on different surfaces. The fundamental physics of the interactions of the solid interface with the ice is not well understood. Nature has, however, dabbled in the prevention of water freezing and fish in cold waters have antifreeze (glyco)proteins, AF(G)Ps, enabling them to survive the low winter temperatures. The AF(G)Ps inhibit the growth of ice crystals by adsorption. The precise function of the AF(G)Ps is not understood but functional groups which are believed to be of importance were mimicked in the models in paper IV and V.

Results from paper IV

A model system was designed and assembled using thiol analogues to mimic crucial parts of an antifreeze glycoprotein (AFGP). The AFGPs consist of a repeating tripeptide unit of Ala-Ala-Thr, where a disaccharide, 1→3 linked 3-*O*-(β-D-galactosyl)-D-*N*-acetyl-galactosamine, is attached to the threonine, with minor sequence variations. The carbohydrate portion of the AFGPs is necessary for activity and thus a Gal(β1-3)-GalNHAc-terminated alkanethiol was synthesized, see figure 34. A methyl-terminated alkanethiol was chosen to mimic the hydrophobic peptide backbone constituted by the methyl groups of the alanine and threonine residues. The alanine and glycosyolated analogues were used to build the surface model. The resulting SAMs were characterized with null ellipsometry, contact angle goniometry, XPS, and infrared spectroscopy.

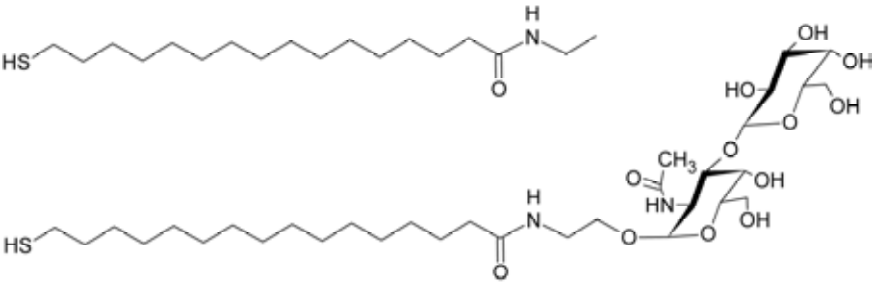

Fig. 34. Molecules used in the SAM model of AFGP, methyl-terminated (top) and disaccharide-terminated (bottom).

The methyl-terminated thiol formed well-ordered monolayers with the alkyl chains in an extended all-*trans* conformation. The disaccharide-terminated thiol formed monolayers with a slightly smaller thickness than expected, the monolayers contained fewer thiols and the alkyl chains were not in an extended all-*trans* conformation. The *gauche* defects appeared most likely from a mismatch between the cross-sectional area of the disaccharide moiety and the optimal pinning distance of alkanethiols on gold.

The mixed monolayers indicated a difference between the two thiols in that the disaccharide compound adsorbed preferentially on the gold surface. This was evident from contact angle, IRAS and XPS measurements and is probably caused by the significantly poorer solubility of the disaccharide thiol compared to the methyl-terminated one. When the bulky disaccharide was mixed with a substantial amount of the smaller thiol (≤ 0.8 mole fraction) the monolayers were well-ordered with an extended all-*trans* conformation. For higher fractions of disaccharide the SAMs became thinner, less densely packed and more disordered.

Initial assessment of the AFGP model was performed in a macroscopic study of the water crystallization temperature using a standard optical microscope equipped with a cooling stage. The temperature of the substrate was lowered, water condensed and then crystallized into ice. As a reference, a well-characterized set of monolayers exposing a mixture of methyl and hydroxyl groups were used to account for differ-

ences caused simply by differences in surface energy. Overall, a dependence of the crystallization temperature on the surface energy was observed, the temperature decreased with increasing methyl content in the SAM. A plateau at ~-6°C was observed for the AFGP model surfaces with a molar fraction of disaccharide > 0.3. This was not observed for the reference surfaces of similar surface energies. The region with deviating behavior is centered at a surface composition of disaccharide of ~0.3, the same content as in the active domain, the tripeptide unit, of the AFGP.

Results from paper V

A model system was designed and assembled using alkanethiol analogues to mimic crucial parts of an antifreeze protein of type I (AFP I). The currently proposed binding face of the AFP I displays threonine (hydrophilic and hydrophobic) and alanine (hydrophobic) residues. Two alkanethiols, an alanine and a threonine analogue, were used to form the model to mimic the AFP, see figure 35.

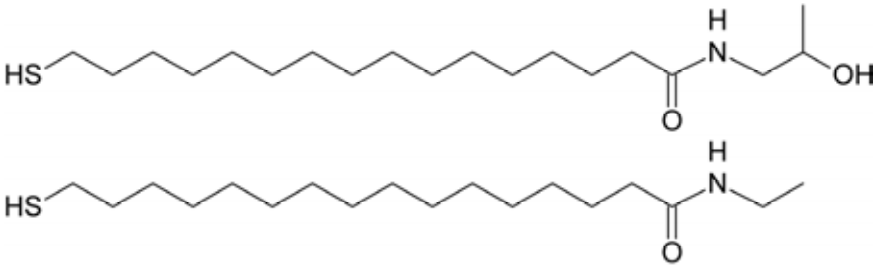

Fig. 35. Molecules used in the SAM model of AFP type I, threonine (top) and alanine (bottom) analogue.

The thiols formed mixed monolayers with the alkyl chains in an all-*trans* conformation. In the case of the alanine and threonine analogues, the composition of the SAM was almost identical to the composition of the solution, which was expected since they are similar in chain length, solubility, and bulkiness of the tail group. The contact angles did, however, show a deviation in that the monolayers appeared

to be more hydrophilic than what would be expected from the composition. Notably, the contact angle for SAMs of the alanine analogue was slightly lower than expected for a methyl-terminated thiol. This could be caused by interactions between water and the amide group.

A detailed study of the ice structure on the monolayers was done by temperature programmed desorption mass spectroscopy in combination with infrared spectroscopy in ultra-high vacuum. The results were in agreement with earlier studies of systems with similar surface energy. Interactions were observed between the water molecules and the amide bond, in good agreement with the contact angle results. Initial assessment of the AFP model at ambient conditions was performed by studying the water crystallization temperature using a standard optical microscope equipped with a cooling stage. The same reference system as in paper IV was used. No differences between the two systems were discernible. A reason could be that it is necessary to have a specific repeating sequence of regularly spaced alanine and threonine residues to interact with the water, which the mixed SAMs do not provide. However, considering the small freezing temperature decrease (< 1°C) caused by AFP I, it is possible that any differences between the reference and model systems would be too small to resolve for this methodology.

Future outlook

There are still many interesting ideas left to explore regarding the model systems described in this thesis. One example is the application of the phosphorylated surfaces in the field of biomineralization by studying the formation of hydroxyapatite. Two other areas of interest are biosensors and the study of water at an interface.

Biosensors

This area includes the development of sensors for phosphatase and kinase and the study of them. One first step could be to study mixed SAMs from amino acid analogues with and without phosphate see how small fraction of phosphorylated amino acid analogues it is possible to detect with electrochemical methods.

Another interesting idea to explore is if it is possible to use phosphorylated amino acid analogues for metal ion sensing. Further studies would be needed to investigate the recognition and possible selectivity of the metal ions.

Supported lipid bilayers are popular models of cell membranes with a potential to be used in biotechnological applications such as biosensors. In a future study they could be used to investigate transmembrane events and cell surface interactions.

Water at an interface

It would be interesting to do a detailed study of the ice structure on the AFGP model system with temperature programmed desorption mass spectroscopy in combination with infrared spectroscopy in ultra-high vacuum as was done with the AFP I model system. Further studies on so-called interfacial water on the phosphate model systems are also of interest to study with these techniques.

At ambient conditions it would be interesting to compare the ice crystallization temperatures for the AFGP model system with surfaces exposing other sugar residues. The experimental setup could probably also be improved with thinner water layers and by using IR to study the freezing of water.

Another possible study would be to use gold nanoparticles to compare the model systems with the antifreeze (glyco)proteins in solution. The reverse, to attach proteins, for example AFP type I, to a gold surface and study the ice crystallization temperature is also interesting.

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