DNA-surfactant complexes as a biomaterial coating

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DNA-SURFACTANT COMPLEXES AS A
BIOMATERIAL COATING

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Voor mijn ouders.
DNA-based biomaterial coatings

Abstract

This chapter is a general introduction and describes the thesis background and outline. A brief description of the biomaterial field is presented and divided into two different trends: the generation of bioactive materials and the construction of bioactive coatings. Due to the complexity of the issues in the development of new biomaterials, which should be biodegradable, bioactive and have the desired mechanical properties all incorporated into one material, the bulk of the implanted materials is still only biologically inert or resorbable at best. The generation of bioactive coatings might form a solution to this problem in the sense that existing implant materials can still be used, but are made bio-compatible using a coating. DNA can be regarded as a low or non-immunogenic anionic bio-polymer and might therefore be a suitable component when a biocompatible coating is developed. Since DNA is highly soluble in water and susceptible to degradation of nuclease enzymes, a coating method has to be chosen which immobilizes DNA onto the surface, thereby reducing its solubility and degradation. Several possible coating techniques are discussed in this chapter.
1.1 Biomaterials

With the aging of the population, the demand for implants is becoming larger and subsequently also the health problems accompanying them are becoming more important. Biomaterials have been defined as substances, other than foods or drugs, used in therapeutic or diagnostic systems. Early biomaterials include metals, wooden tooth and glass eyes, which were eventually replaced by materials that better matched the physical properties of the replaced tissue, with a minimal toxic response. A common feature of these first generation biomaterials was their biological “inertness”. However, when a larger understanding of the immune system was developed, many implanted materials were found to elicit still an inflammatory response of the human body against the foreign implant, interfering with the wound healing process and in severe cases even resulting in failure of the implant.\(^4\)\(^6\) As a result, the field shifted towards the production of second-generation biomaterials, which emphasised the development of bioactive components rather than inert materials, in order to promote a favourable reaction of the physiological environment towards the implanted material leading e.g. to direct cell differentiation or mineral formation. A different class of second-generation biomaterials focussed on resorption of the implanted material, which eventually had to be replaced by regenerated tissue.\(^8\) The biomaterial field is currently developing a third-generation of biomaterials, which combines both above mentioned concepts of second-generation materials in the formation of resorbable bioactive materials.\(^8\)\(^10\) These can be used for tissue engineering, in which progenitor cells are seeded onto modified resorbable scaffolds outside the human body and become differentiated into naturally occurring tissue. The construct is then implanted in the human body and is replaced by new tissue after resorption of the scaffold. In addition, *in situ* tissue regeneration can be employed, in which resorbable bioactive biomaterials in the form of powders, solutions, gels or doped microparticles stimulate local tissue repair via the release of e.g. growth factors or other signalling molecules by diffusion or network breakdown.\(^8\) Polymers have been extensively studied for use in the biomaterial field because they are highly versatile in tuning the material properties by variation of e.g. monomeric building blocks, molecular weight or use of copolymers and additives.\(^12\)\(^14\) However, these materials are mostly only biodegradable and functionalization is limited to both doping and mixing with additives or covalent modification.\(^16\)\(^17\) Mixed or doped systems lead to dynamic systems, which in many cases are unstable, whereas stable covalently modified systems often lack the ability to adapt to the environment and have synthetic limitations. Supramolecular chemistry can be used in order to produce third-generation biomaterials that show both a high degree of synthetic versatility and
stability. By using more dynamic interactions like hydrogen bonding or disulfide bonds, various building blocks can be linked together, resulting in the formation of a multitude of supramolecular materials. The implementation of supramolecular chemistry in the field of polymer chemistry may lead to the generation of new and highly tunable polymeric biomaterials, which through non-covalent interactions can be modified with e.g. peptide fragments, resulting in stable bioactive and biodegradable scaffolds. An example of this approach is the modification of low molecular weight polymer segments with ureido-pyrimidinone (Upy) units, which dimerize via strong quadruple hydrogen bonds, resulting in the formation of a supramolecular polymer that can be modified with various short peptide sequences also equipped with a Upy unit. Another intriguing modular approach in the generation of functional supramolecular constructs is shown by the group of Stupp et al., who developed a large number of amphiphilic molecules, which are composed of various signalling peptide sequences linked to an alkyl tail and self-assemble into nano-fibres depending on the pH, forming a bioactive gel.

Although very promising, the large scale implementation of third-generation biomaterials within the medical field is still far away and the majority of the implanted materials is still of the biological inert nature or at best resorbable, e.g. sutures. The generation of materials that are bioactive, while at the same time showing all the desired mechanical material properties is, also due to lack of knowledge, extremely difficult and replacement of existing implant materials will most likely not be achieved in the near future. To solve this problem, a different approach can be employed: the application of bio-active coatings on existing implant materials.

1.2 Biomaterial coatings

Since bulk properties largely determine the suitability of an implant material for a certain application and the biological response is mainly determined by the biomaterial surface, via the interactions with components of the biological surroundings, the application of a coating material can be a short term solution to a complex problem. By using a bioactive coating, existing implant materials, which have been developed over many years with the objective of optimizing the mechanical properties, can be improved with respect to the immune response and tissue integration. An example is the hydroxyapatite* (Ca_{10}(PO_4)_6(OH)_2) coating, which stimulates the formation of bone firmly attached to the implant. The coating is currently

* the major mineral constituent of bone
commercially applied using the plasma spray technique and these hydroxyapatite coated implants have become widely used over the last twenty years, with several companies manufacturing devices for orthopedic (e.g. hip-prostheses, Figure 1A) and dental applications. Another example of a commercially available bioactive coating is the coronary artery stent (Figure 1B), which is coated with medication (sirolimus or paclitaxel) to prevent restenosis of the artery.

This thesis focuses on the use of deoxyribonucleic acid (DNA) (Figure 2) as a coating material. Although most well known as a carrier of genetic information, the structural properties of DNA give this unique biomacromolecule a great potential for use as a biomaterial coating. The molecular structure of DNA in vertebrates is homogeneous, and the non- or low immunogenic properties of DNA (compared to other biological antigens like proteins and sugars) may reduce both innate and acquired immune responses. Additionally, DNA can incorporate other molecules via groove-binding and intercalation, which creates opportunities to specifically deliver desired biological mediators in the direct vicinity of the implantation site. Finally, especially for hard tissue implant applications, the high phosphate content in DNA might, via the high affinity of phosphate for calcium ions, beneficially affect the deposition of calcium in the bone formation process. The use of DNA as a functional biomaterial, instead as a carrier for genetic information, has only recently been suggested, and pioneering efforts by Okahata et al. have resulted in the fabrication of a DNA-containing bulk (bio)material, which was demonstrated to cause no adverse reactions upon subcutaneous implantation in the back of rats. The application of DNA as a coating material,
However, can be expected to be hampered by its easy nucleolytic degradation and its solubility in aqueous solutions. The DNA-containing bulk material of Okahata et al. was found to easily detach from different types of substrates. Other methods to obtain stable DNA-containing coatings for biomaterial purposes, therefore, have to be explored.

**Figure 2.** DNA. A. CPK-model of the double stranded DNA helix. B. Molecular model highlighting the internal basepair structure and the backbone. C. Lewis-structure of a single DNA-strand.

### 1.3 Coating techniques

Various coating techniques can be employed to deposit polymer material onto a suitable substrate. A coating technique is often selected on the basis of a number of criteria, including simple machinery, easiness in use and high surface coverage and homogeneity, which also relates to high tolerance with respect to substrate and coating material and to substrate morphology. Roughly three coating techniques can be distinguished: dip, spray and spin coating.
The latter technique is widely used in industry and therefore offers the advantage of large-scale production. However, in order to produce a homogeneous film, the technique requires a quick spreading and evaporation of the solvent, which imposes limitations in the use of aqueous media. This poses a problem for the use of biological components, since most molecules of biological origin are only soluble in water or denature in organic solvents from which spin coating is possible. A way to solubilize DNA in organic solvents has to be found if the spin coating technique is to be applied for DNA-based coatings.

The spray coating technique knows many variations of which two are currently most applied in the field of biomedical coatings: plasma spraying and electrostatic spray deposition (ESD). Plasma spraying, as mentioned above, is used for the deposition of hydroxyapatite coatings, however this technique requires high temperatures, which makes it only suitable for mineral components that are able to withstand the high operating temperatures. If additives of biological origin need to be incorporated, other techniques have to be used, since all organic molecules will decompose when introduced into a plasma. The ESD technique uses a high voltage to produce very fine droplets, which are deposited onto the substrate. Similar to the spin coating technique, this imposes limitations in the use of solvents and also in this case addition of organic solvents like ethanol is necessary to produce a homogeneous film. Both spin and spray coating techniques have an additional disadvantage if they are applied on biomaterial implants, since an increasing number of implanted scaffolds are porous in nature and these coating techniques predominantly cover the outside of the material. Dip coating techniques are therefore better suited, as they cover also the inside of interconnected porous materials. Two techniques have been used in this thesis: the Layer-by-Layer (LbL) technique and the Langmuir-Blodgett film deposition.

The Langmuir-Blodgett technique is based on the formation of a close packed monolayer (often composed of surfactants) at the air water interface, via spreading of the desired compound on water and subsequent compression of the molecules using a Langmuir-trough. While at constant surface pressure, depending on the substrate polarity or the desired film structure, the film is deposited via vertical dipping (Figure 3A) starting either above or below the air-water interface, resulting in a layered architecture. Horizontal transfer, known as Langmuir-Schaefer deposition, can also be applied and repeated several times to increase the layer thickness. DNA is a negatively charged polyelectrolyte and has therefore been used in conjunction with cationic surfactants in the formation of DNA-surfactant monolayers. In these systems, DNA is bound underneath the surface of a close packed surfactant monolayer and when deposited on a suitable substrate may also be used to form DNA-based coatings, in which the cationic amphiphillic molecules can act as an immobilization agents.
Since its introduction by Decher,\textsuperscript{47,48} the LbL self-assembly technique has received a great deal of attention as a versatile and simple coating technique, which does not require large machinery. It involves the alternate absorption of two attracting components theoretically resulting in a layered structure. LbL deposition is mostly applied in the case of oppositely charged polyelectrolytes and is then also referred to as the electrostatic self-assembly technique (ESA) (Figure 3B), however, it can be used for other systems as well, as long as the two components adhere to each another. Due to the layered structure, LbL films can act as a multi-compartment drug delivery films, making them ideal candidates for use in the field of biomaterial coatings. DNA, also a polymeric anionic polyelectrolyte, has already been used in the LbL deposition with cationic polyelectrolytes as positively charged counterparts,\textsuperscript{49-56} however, mostly related to DNA-based sensors or transfection but not yet with the aim of producing a bioactive coating.

1.4 Aim of the thesis

Vertebrate DNA can be regarded as a low or non-immunogenic anionic bio-polymer and it was therefore speculated that implants, coated with DNA, would show a reduced inflammatory response.\textsuperscript{30-33} Since DNA is highly soluble in water and susceptible to degradation by nuclease enzymes, a coating method has to be chosen which immobilizes DNA to the surface, thereby reducing the solubility and degradation. Since only the surface of the coating is in direct contact with the cells it is imperative that DNA is situated on the outside of the film. In addition, the ability to functionalize the coating with molecules of biological origin
is to be investigated in order to produce a versatile bio-active coating for both drug delivery and surface signalling. Finally, it is of interest to study the possibility of mineral deposition on the surface of the coating in order to produce a coating that is also suitable for hard tissue implants.

1.5 Outline of the thesis

In chapter 1 some background information and the aim and outline of the thesis is presented. Since, as will be described in this thesis, the eventually developed coating is based on the LbL film deposition technique, with DNA as the anionic and a bis-ureido based surfactant aggregate as the cationic component, a literature overview on the LbL technique and the bis-ureido group as a supramolecular stabilization and modular functionalization unit is given in chapter 2. Chapter 3 briefly discusses initial studies to use polymerizable DNA-surfactant Langmuir-monolayers and spin coated DNA-surfactant complexes for the formation of DNA-based biomaterial coatings. Both approaches proved to be irreproducible and unsuitable for application. The LbL technique was chosen, therefore, as a method to immobilize DNA on the surface of implant materials, e.g. titanium. The initial coatings are based exclusively on polymeric components, in which poly(allylamine hydrochloride) (PAH) and poly-D-lysine (PDL) are used as cationic components and DNA as the anionic component. Chapter 4 describes the construction and chemical analysis of these films as well as a summary of the biological assays carried out in collaboration with the Department of Periodontology & Biomaterials of the Radboud University Nijmegen. Analysis of the polymer-based coatings showed an enrichment of DNA at the surface of the films, however, also showed mixing of the individual layers. As is described in chapter 2, polymeric LbL films are subject to internal diffusion of polymer chains, which results in mixed coatings. In order to construct a “truly” layered film on a nanometer scale, diffusion had to be controlled by replacing the cationic polymer component. Chapter 5 introduces a bis-ureido based cationic surfactant, which due to a combination of hydrophobic and hydrogen bonding forms stable ribbon-like bilayer aggregates in water. It was supposed that these aggregates would limit the diffusion of DNA chains and form a structure similar to the lamellar phase of DNA-cationic surfactants complexes. The formed aggregates have been extensively studied and the results are described in this chapter. It is shown that their shape and size can be tuned by varying the temperature and concentration at which they are formed. Furthermore, it is shown that the bis-ureido based aggregates do not behave like a classic surfactant system but possess properties similar to crystals. The presence of the bis-urea units within the aggregate structure opens up possibilities
for modular functionalization with similar bis-urea containing molecules. Chapter 6 shows the possibility of anchoring a dye and a compound of biological origin (biotin) to the ribbon structure using the self-recognition capabilities of matching bis-urea groups that had been coupled to the incorporated molecules. To study the interaction of the bis-ureido based surfactant with DNA, Langmuir experiments have been performed which are described in chapter 7. These experiments show that injected DNA cannot penetrate a preformed surfactant monolayer on water, indicating that the ribbon aggregates are stable when in contact with a DNA solution. However, these experiments also revealed that no DNA-surfactant monolayer† is formed when the surfactants are spread on top of a DNA containing subphase. The final chapter 8 combines the preceding chapters and discusses the construction of a truly layered LbL coating on a nanometer scale, in which DNA is used as the anionic and the ribbon aggregates as the cationic component. It is also demonstrated in this chapter that individual aggregate layers can be functionalised with biotin and preliminary biological assays showing an increase in cell proliferation are presented.

1.6 References


† Ordered DNA molecules attached underneath a close packed amphiphillic surface


Layer-by-Layer assembly and molecular recognition

Abstract

Two fields of research are of particular importance to this thesis: Layer-by-Layer (LbL) assembly and materials designed by molecular recognition. In this chapter two literature overviews are presented, in which the first part focuses on the Layer-by-Layer (LbL) film deposition technique aiming at biomaterial and biomedical applications. The build-up characteristics of LbL films are described with particular attention for the formation of either layered or mixed structures and the potential implications of the resulting degree of mixing for the intended biomaterial application. In addition, examples will be presented in which a variety of different biological assays are performed on a multitude of LbL films. The second part concentrates on molecular recognition based on hydrogen bonding motives.
2.1 Layer-by-Layer self-assembly

For about 65 years the controlled fabrication of nanostructured films has been dominated by the Langmuir-Blodgett (LB) technique, which ensures separation of the individual layers down to the molecular level.\(^1\) However the LB technique is limited to flat substrates and restricted to at least one component having surface active properties. The principle of multilayer self-assembly was first described by Iler as early as 1966.\(^2\) Although other groups claim to be the first to use the multilayer self-assembly technique,\(^3\) it was the group of Decher who initiated systematic studies on this technique in the early nineties.\(^4,5\) By the late 1990’s the “Layer-by-Layer” (LbL) technique, as it was then called, had received considerable attention from physicists, chemists and even scientists from the biomedical field due to its simplicity in construction, combined with its versatility in components.\(^6\) In general, the LbL self assembly technique can be applied to any two or more adhering components, by using alternate absorption from any solvent onto a suitable substrate (see Figure 3B, Chapter 1.3). In most cases it has been applied to polyelectrolytes and is thus also referred to as the electrostatic self-assembly (ESA) technique. Since the beginning of this millennium the application of the LbL technique both in the biomaterial and biomedical field has increased significantly and many research groups are exploring the possibility of using especially polyelectrolyte multilayers (PEM) as biomedical coatings and drug delivery systems. The groups of Möhwald and Sukhorukov are specialized in constructing microcapsules based on the LbL technique with the aiming at drug delivery.\(^7,17\) However, since this thesis is focussed on DNA-based coatings, this literature overview will focus on the use of the LbL technique aimed at the construction of biomaterial coatings.

<table>
<thead>
<tr>
<th>Cationic polymers:</th>
<th>Anionic polymers:</th>
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<tbody>
<tr>
<td>CHI : Chitosan</td>
<td>HA : Hyaluronan/Hyaluronic acid</td>
</tr>
<tr>
<td>PAAm : Poly(acrylamide)</td>
<td>PAA : Poly(acrylic acid)</td>
</tr>
<tr>
<td>PAH : Poly(allylamine hydrochloride)</td>
<td>PLGA : Poly(L-glutamic acid)</td>
</tr>
<tr>
<td>PDL : Poly(D-lysine)</td>
<td>PMA : Poly(methacrylate)</td>
</tr>
<tr>
<td>PEI : Poly(ethylenimine)</td>
<td>PSS : Poly(styrene sulfonate)</td>
</tr>
<tr>
<td>PLL : Poly(L-lysine)</td>
<td></td>
</tr>
</tbody>
</table>

Tabel 1. Polymer abbreviations.
2.1.1 The LbL assembly mechanism

The successful application of the LbL film deposition technique in the field of biomaterial coatings is dependent on three factors: surface composition, film degradation and compartmentalization, of which the last two are mainly concerned with drug release. Since only the outer surface of the coating is in direct contact with the surrounding tissue, the surface composition in the top nanometers of the film will largely determine the biological response towards the coating. Control over the surface composition is therefore of paramount importance for biomaterial applications. Since the LbL deposition in theory yields a layered film, many researchers have investigated the possibility of drug delivery from enriched individual layers. The drugs may be released either by degradation of the coating down to the functionalized layer, or by slow release from an enriched compartment. For both release mechanisms, the degree of layer separation is the most important parameter to control. Both parameters, surface composition and layer separation, are dependent on the diffusion rate of the components used.

Since its introduction, many physicists and chemists have debated on the degree of separation in LbL films and tried to compose a mechanism for the LbL build-up.\textsuperscript{1} Today, for polyelectrolyte components, this mechanism is fairly well understood. Two different growth curves are known: linear growth and exponential growth, both of which are controlled by the rate of diffusion of the individual components in the film. More precisely, linear growth can be regarded as a specific case of exponential growth in which diffusion is limited.

The build-up mechanism can be described in terms of two compartments separated by a semi-permeable wall (Figure 1).\textsuperscript{18} The left compartment will be the LbL film, whereas the right compartment is either the dipping or the washing solution. When the substrate is immersed in one of the polyelectrolyte solutions (e.g. the negatively charged component), the right compartment is filled with negatively charged polymer chains. Due to the difference in chemical potential, the polyelectrolytes will diffuse from the right to the left compartment (Figure 1A). During the diffusion process the negatively charged polymers will also adhere to the semi-permeable wall (or coating surface). The diffusion of polymers will continue until either equilibrium is reached, or the repulsion of the negatively charged barrier, resulting from adsorption of the polyelectrolytes to the semi-permeable wall, prevents further diffusion. During the washing step, following the first deposition step, the right compartment is filled with water (Figure 1B). The situation is reversed and the chemical potential in the left compartment (the LbL film) is larger than that in the right. Now, negatively charged polyelectrolytes will diffuse back, out of the left compartment, unless the negative charge
barrier, resulting from polymer adsorption in the previous step, is too high and prevents diffusion (Figure 1C). After the washing step, the substrate is immersed in the polymer component of the opposite charge (i.e. positively charged). The positively charged polymers will immediately adhere to the negative surface of the semi-permeable wall (Figure 1D).

**Figure 1.** Schematic representation of one build-up cycle for exponential and linear growing LbL films, using a model containing two compartments separated by a semi-permeable wall. First step: adsorption of the negative polyelectrolyte to the surface of the wall (A,G), accompanied by diffusion into the left compartment for exponential growth (A). Second step: washing with water (B,H), accompanied by possible diffusion of the negatively charged polyelectrolytes out of the left compartment for exponential growth (C). Third step: deposition of the oppositely charged component onto the present previous layer (D,I). Inversion of charge and removal of the charge barrier results in diffusion of the remaining oppositely charged “free” polyelectrolytes from the left compartment to the right resulting in immediate complexation at the surface interface for exp. growth (E). Final result: additional increase in film thickness for exponential compared to linear growth (F,J).
However, the positive right compartment will also act as a sink for the mobile negatively charged polymers still present in the left compartment. The negative charge barrier between the two compartments will be instantly removed by absorption of positive polymer chains, thus all remaining negatively charged polymer chains in the left compartment will diffuse rapidly towards the positive right compartment. At the surface of the semi-permeable wall they will be complexed by the excess of positively charged polyelectrolytes resulting in added mass (Figure 1E). The cycle is then repeated, however as the film thickness increases so does the volume available for diffusion (Figure 1F). If by comparison the left compartment becomes larger, more chains can diffuse into this section when the charge barrier repulsion still allows it. As a result more chains can diffuse out of the left compartment and thus out of the film upon charge reversal. The added mass on the film surface upon complexation of the diffusing chains is therefore directly related to the diffusion volume and thus the film thickness. A thin film (at the beginning of the LbL deposition) represents a small diffusion volume yielding a limited amount of added mass upon complexation, whereas a thick film (at later stages of the deposition) represents a large reservoir of diffusing chains and thus a large potential amount of added mass. The result is an exponential growing film, which is dependent on the degree of diffusion. Theoretically, if the charge barrier at the film surface is reached before diffusion occurs, the film will only grow as a result from direct adsorption of oppositely charged polymer chains at the surface (Figure 1G-J). This will result in a linear growth profile, which makes linear growing films a special case of diffusion limited exponential growth.16-20

The above described mechanism is only valid when diffusion plays a role for one of the two components. If both components are able to diffuse, the situation becomes extremely complicated,21 since diffusion of one component into the film will be accompanied by diffusion of the other component out of the film. Furthermore it should be mentioned that the above model does not take penetration depth into account. Diffusion can be limited by the presence of a charge barrier, however, it can also be limited by the ability of the polymer chains to penetrate the film structure. If the penetration depth is a fixed distance, exponentially growing films will change into linear growing films upon reaching a film thickness equal to the penetration depth, since the effective diffusion volume is limited to that distance and remains constant.22,23 Nevertheless, it is clear that any form of diffusion will result in mixing of the layers on a molecular level. Especially, complexation of the outward diffusing chains with incoming polyelectrolytes will result in a mixed film composition in the top nanometers of the coating. The exact molecular composition on a nanometer level at the surface of the film is still a virtually unexplored area, yet it is this region that is most important to biomaterial applications. Although zeta-potential measurements, AFM and contact angle measurements
are frequently employed, these techniques do not provide a molecular profile. A recent publication by Hwang illustrates the importance of surface composition to the biomaterial field. In this paper it was shown that PLL/HA polyelectrolyte multilayers (PEM) induced cell death in monocytes irrespective of the layer termination (either PLL or HA), whereas only PLL had been shown to induce cytotoxic effects on its own. Moreover, it was confirmed by time-of-flight second ion mass spectrometry (ToF-SIMS) that no significant difference in surface chemistry existed for either HA or PLL terminated films. This suggests that mixed films are obtained due to PLL chain diffusion to the surface explaining the observed cytotoxicity in all cases.

Another example indicating the presence of a mixed surface structure involves the nucleation of calcium phosphate crystals on the surface of polyelectrolyte multilayers. The linear growing PAH/PSS multilayer was demonstrated to give oppositely charged surfaces depending on layer termination. Nevertheless, both types of surface layers induced calcium phosphate nucleation although, according to the literature, such an effect should only be expected on negatively charged surfaces.

Following the above argumentation, the degree of layer separation can be viewed as a measure for diffusion and thus for mixing. The formation of well-defined separate layers might therefore be used as an indication that the surface experiences a lesser degree of mixing. It has been demonstrated by Lavalle et al. that indeed exponential growing films are subject to diffusion, as was evident from confocal laser scanning microscopy (CLSM) in which fluorescently labelled PLL incorporated in a single layer, however, was observed to be present throughout the complete film after LbL deposition of additional double-layers (Figure 2). Moreover they demonstrated that linear growing films could act as barriers between exponentially growing films, preventing the fluorescently labelled component to spread beyond the linear growing film (Figure 2D). Although linearly growing components like HA show only a narrow band when a single layer of fluorescently labelled polymer is incorporated in a PEM, its layer thickness is still within the order of several hundreds of nanometers.

Several reports claim, based on X-ray diffraction techniques or the presence of intact secondary structures in polypeptide PEMs that for linear growing films well ordered individual layers are formed. Nevertheless, it is still generally believed that all organic multilayers are subject to some degree of diffusion. This entails that most, if not all, currently studied PEMs for use as a biomaterial coating have a mixed surface. Nevertheless, many excellent papers, which will be discussed in the following section, have been published on the subject mostly showing a promising biological response. A variety of different cell types have been cultured on various PEMs illustrating their biocompatible nature.
Figure 2. Vertical sections through different film architectures containing labeled polyelectrolytes (PLL-green; HA-red) obtained from CLSM observations. A. [PLL/HA]₁₉-PLLₙ multilayer containing two labeled layers, PLL₁₉-green and HA₁₉-red (subscript denotes double-layer number). Green fluorescence is visible over the total thickness of the film indicating complete diffusion of PLL. B. [PLL/HA]₂₅ multilayer containing two labeled layers, PLL₁₉-green and HA₁₉-red. Green fluorescence is visible over a total thickness of the film indicating total diffusion of PLL, however red fluorescence is only visible in ~1 μm indicative of limited diffusion. C. [PLL/HA]₂₀ multilayer in which the 14th and 20th HA layers are labeled red and the last PLL layer has been labeled green. D. Exponential growing PLL/HA-multilayers separated by linear growing PAH/PSS-multilayers that act as barriers for diffusion of fluorescently labeled PLL.²⁹,³⁰

2.1.2 LbL biomaterial coatings

Although many research groups involved in LbL deposition have also considered the possibility of biomaterial applications, systematic studies in this area have been generated by the groups of Lavalle, Voegel and Schaaf, of which most in vitro and in vivo experiments originate from the last 3 years. This field of research is therefore just starting; however, the number of scientific publication is already impressive considering the short period. The various generated biomaterial coatings can be divided into two groups: films of which the coating
components facilitate a biological response and films of which a certain layer is functionalized or enriched with a bioactive component. Combinations of the two approaches also exist, however, when coating enrichment is involved it will herein be classified as such.

2.1.2.1 Biologically active coating components

The control over cell adhesion is essential when LbL films are to be used in a biomaterial coating. Depending on the application, cells should either adhere more strongly to the coated surface e.g. to promote wound healing, or show no adhesion e.g. for coatings deposited on vascular implants like artificial heart valves. Zhu nicely demonstrated increased chondrocyte proliferation on 3-D scaffolds, which had been coated with PEI/gelatine multilayers. Richert, Vautier and Zhang investigated cell adhesion properties of chondrocytes and chondrosarcoma cells on PLL/PLGA, PLL/HA, CHI/HA and collagen/HA multilayer films in which the effect of layer termination, cross-linking, pH and salt concentration during multilayer build up were studied. It was observed that the cell attachment on the multilayer films was dependent on the layer thickness and swellability, which both can be tuned using pH and salt concentration. Furthermore, it was shown that for thick and cross-linked films the cell adhesion was independent of layer termination and that especially cross-linked films show improved cell adhesion of chondrosarcoma cells, whereas the native films were non-adhesive. In contrast Yang demonstrated that cross-linked PAAm/PAA and PAAm/PMA exhibit a high resistance to adhesion of mammalian fibroblasts.

It is important to reduce cell adhesion for e.g. stent applications to prevent restenosis of the artery, however, since the vascular wall is composed of endothelial cells (EC) a selective single layer of ECs might be preferred. Kerdjoudj demonstrated that EC attachment to PAH/PSS multilayers is reduced, whereas, Boura showed improved cell attachment and viability of EC’s to PAH/PSS and PDL/PLGA double-layer terminated films. Importantly Thompson showed that the mechanical compliance of PAH/PAA and PAH/PAAm PEMs affects the EC attachment more strongly than did the ionic character of the terminating layer and that EC attachment can be regulated by varying the mechanical compliance of the coating.

Salloum and Olenych investigated the effect of surface charge and hydrophobicity on the adhesion, morphology and motility of rat aortic smooth muscle cells (SMC) using a multitude of different PEM’s. It was found that hydrophobic surfaces promoted cell attachment regardless of surface charge, whereas hydrophilic surfaces reduced cell adhesion, which was even more pronounced when the surface charge was increased. Micro-patterning of
hydrophobic PEMs between hydrophilic multilayers showed a dramatic preference for cell adhesion and spreading onto the hydrophobic domains. In contrast, silicon rubber substrates coated with hydrophilic fibronectin/PDL or laminin/PDL showed an increased nerve cell adhesion compared to the bare hydrophobic substrates.\textsuperscript{55,56} Additionally, Kommireddy showed that super-hydrophilic (water contact angle <10\(^\circ\)) multilayer films composed of titanium dioxide nanoparticles and PSS, were biocompatible with human dermal fibroblasts.\textsuperscript{57} The same films promoted attachment, proliferation and spreading of mouse mesenchymal stem cells, which was also dependent on the surface roughness.\textsuperscript{58} Adhesion of blood platelets and blood coagulation was demonstrated to be reduced on albumin/heparin\textsuperscript{3} and chitosan/dextran\textsuperscript{59} multilayer films.

The above presented literature overview contains several seemingly contradictory results. While one group observes improved cell adhesion to a particular film, another observed the opposite effect in coatings comprised of a similar composition. Due to the mixed character of the coatings and the large variety in components and used cell types, it is not yet possible to see a clear trend and to predict the effect of a specific LbL parameter, e.g. pH during build up, cross-linking or layer termination on the overall biological effect. In some cases layer termination does seem to play a role. For example, PEI layer termination is cytotoxic for osteoblast-like and human periodontal ligament cells,\textsuperscript{60} although PSS, PAH and PDL termination is not. In other cases, a similar biological effect on cells was observed irrespective of layer termination, e.g. monocyte activation and complete cell death on both PLL and HA terminated layers of PLL/HA multilayer films (Figure 3A).\textsuperscript{26} In contrast, modification of a PLL terminated layer with alpha-melanocyte stimulating hormone (\(\alpha\)-MSH) that was covalently bound to PLGA, did not result in monocyte cell death, indicating that the surface composition changed and that PLL was not able to diffuse past the added modified PLGA layer.\textsuperscript{61} The above examples illustrate that it is very difficult to relate layer termination to a certain biological effect and to predict the outcome of these type of experiments. This is due to the fact that in most cases the exact chemical composition in the top nanometers of the coating is not known and that, due to the possible mixed surface composition, interpretation of biological effects on these seemingly similar coatings remains puzzling.
2.1.2.2 Functionalized and enriched PEMs

Enrichment of PEMs with a biological active component has a twofold application: surface modification and drug delivery. Both applications rely on the control over the degree of diffusion through the multilayer, although in a different way. In the case of surface modification one would ideally want to limit mixing and thereby the diffusion of components, since the biological active groups should be presented to the cells at the surface as much as possible. In contrast, drug delivery is dependent on diffusion in order to release the active components. Finally, a special position is reserved for timed release, since this method would require specific limited diffusion in-between time points where the drug needs to be released. This latter approach can be pursued through controlled degradation of the coating combined with selective modification of predetermined layers.

Figure 3. A. Live/Dead® assay of adherent monocytes on PLL- and HA-terminated PLL/HA-multilayer surfaces after 2 days showing predominantly dead cells (red) on the coated substrates and live cells (green) on the non-coated control.26 B. Observation by CLSM of a multicompartiment film composed of [PLL/HA]30/PLL-green/PLGA/[PLL/HA]30/PLL-red in contact with bone marrow cells. Panels 1 correspond to film x-z sections (in red and green channels), 2 and 3 correspond to x,y sections in the bright field red and green channels, respectively. The white solid line indicates the substrate surface; the white dotted line indicates the film surface. It is shown that after 1 hour only the red labeled PLL is internalized, since the PLGA layer acts as barrier for the green labeled PLL. After 5 days and more pronounced after 10 days also the green labeled PLL is internalized.62 C. Phase contrast micrographs of fibroblasts adhering to RGD functionalized line patterns. D. same as C only block patterns.
To start with the latter, initial degradation experiments of PEMs have been performed \textit{in vitro}, however also a limited amount of reports have described degradation \textit{in vivo}. Although not investigated in the presence of cells, Zhang demonstrated that the time required for the complete erosion of different poly-amine/PSS multilayers in PBS buffer varied between 50 hours and 15 days.\textsuperscript{63} \textit{In vitro} and \textit{in vivo} degradation studies of CHI/HA PEMs have been performed by Etienne and Picart in an oral enviroment.\textsuperscript{64,66} It was found that cross-linking of polysaccharide multilayers reduced both the \textit{in vitro} macrophage and enzymatic degradation as well as the degradation \textit{in vivo} of these PEMs on oral scaffolds implanted in mice and rats.

The possibility of using the combination of exponential growth regimes as drug release reservoirs and linear growth regimes as barriers to form a multi-compartment film has been proposed by Garza as a method to facilitate timed release of drugs upon film degradation.\textsuperscript{29} Unfortunately, it was demonstrated by the same group that synthetic linear growing PEM barriers could not be degraded even by phagocytic cells. Biodegradable PLGA layers, however, could be degraded by bone marrow cells and were still able to act as barriers. It was nicely demonstrated that these cells sequentially internalized red and green labelled PLL, which were positioned in different exponential growth compartments separated by a PLGA layer (Figure 3B).\textsuperscript{62} Also protein A (PA), which induces the production of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-\(\alpha\)), was incorporated at different predetermined depths within a PLL/PLGA multilayer films. The PLL/PLGA coating was alternately composed of \(d\)- and \(l\)-enantiomers, of which the non-biodegradable \(d\)-enantiomers acted as barriers facilitating timed expression of TNF-\(\alpha\) upon film degradation down to the PA loaded compartement.\textsuperscript{67}

The continuous release of bioactive compounds from LbL films mainly by diffusion has been investigated as well for many different active compounds and cell types. Berg showed that micro- and nanoporous PAH/PSS-PAH/PAA multilayer films, which had been loaded with ketoprofen and cytochalasin D, by dipping of the final film in a drug solution, followed a zero-order kinetics release profile for many days. Cytochalasin D retained its ability to induce mitosis in fibroblasts.\textsuperscript{68} Vodouh\`{e} demonstrated the incorporation and release of paclitaxel in PLL/HA multilayers, by which the cell viability could be reduced by 80\% 96 hours after cell seeding.\textsuperscript{69} They also demonstrated that Brain Derived Neurotrophic Factor (BDNF) or Semaphorin 3A (Sema3A) could be incorporated in multi-component PEI,PAH,PLL/PSS,PLGA multilayers and still remained active towards motoneuron growth.\textsuperscript{70} DNA could also be successfully delivered from PLL/PLGA coatings, aided by co-incorporated charged cyclodextrins, to cells in contact with the film.\textsuperscript{71} However, also multilayer films composed of plasmid DNA encoding for enhanced green fluorescent protein (EGFP) and a synthetic biodegradable polyamine were able to induce transfection of EGFP without the aid of
additives. In addition, polyplex embedding, formed by pre-complexed plasmid DNA with PEI, into synthetic and naturally degradable polyelectrolyte multilayers was efficient for transfection of human hepato-cellular carcinoma cells. Nevertheless, cyclodextrines have been shown to act also as chaperones for the anti-inflammatory agent piroxicam and a lipid A antagonist (LAA) that could be incorporated and released from polyelectrolyte multilayers while retaining their biological activity.

Apart from the above described effects on various cell types also studies on the effect of multilayer films on bacteria and fungi have been reported. Boulmedais used PLL/PLGA multilayers, in which the final layers were modified with PLGA grafted poly(ethylene glycol) that showed more than 70% reduction of Escherichia coli (E. coli) bacteria adhesion. However, as also demonstrated by Salloum for adhesion of smooth muscle cells to PEO modified films, the presence of PEG type polymers might invoke reduced adhesion of cells in general, including bacteria. A similar effect was reported by Etienne, who observed 98% inhibition of E. coli growth at the surface of antimicrobial peptide defensin-functionalized multilayer films. However, this occurred only when positively charged PLL was present in the outermost layer of the film. In light of the results as obtained by Hwang where PLL was able to induce cell death, it can be supposed that PLL termination is predominantly responsible for the antibacterial properties and not the incorporated defensin. Nevertheless, it was also shown that insertion of a chromogranin A-derived antifungal peptide into similar PEMs resulted in antifungal activity by interacting with the fungal membrane and in penetration into the cell in vitro. Finally, Lee showed antibacterial properties of silver nanoparticle loaded multilayers by release of silver ions from the multilayer film.

An ingenious way to obtain well defined drug delivery compartments within a multilayer film, without using the individual layers as reservoir, is the embedding of vesicles in PEM and using the vesicle interior as a reservoir. Proof of principle was obtained by embedding giant liposome microreactors within PEMs. The vesicle interior of the liposome reactors was filled with an enzyme, which still was able to trigger precipitation of calcium phosphates within the liposome, while embedded in the multilayer film.

One of the few in vivo studies based on drug release was performed by Schultz, who demonstrated that PLL/PLGA multilayers functionalized by covalent binding of a synthetic analogue of the anti-inflammatory peptide α-MSH remained biologically active for tracheal prostheses implanted in rats. Systemic anti-inflammatory IL-10 production was only detected in rats implanted with prostheses functionalized by α-MSH. Covalent binding of bioactive additives is an approach that is also applied for surface modifications. Picart functionalized PLL/PLGA films with the cell adhesion promoting peptide sequence RGD by grafting the RGD
containing peptide sequence to the PLGA terminating layer and showed that indeed cell adhesion was promoted for the functionalized films. Berg took this approach one step further and constructed patterns of PAH on cell resistant PEM’s via polymer-on-polymer stamping followed by covalent coupling of an RGD containing peptide sequence to these PAH patterns. It was convincingly demonstrated that fibroblasts cells only adhered to the RGD functionalized parts (Figure 3C/D). Chluba showed that a synthetic α-melanocortin derivative, covalently coupled to PLL forming the outer layer of a PLL/PLGA multilayer film, remains as biologically active as the free hormone towards murine melanoma cells. Furthermore, the long time activity of the hormone is maintained when embedded in multilayer architectures, whereas its short time activity depends on integration depth.

Although most of the above reports on film functionalization and enrichment are performed using mixed systems and the exact chemical structure is not known, it is evident that many of the observed biological effects can be related to the incorporated bioactive molecules or functional groups. This demonstrates that the LbL films do have great potential for use as a biomaterial coating, however improved structural and chemical analysis should help to exclude effects originating from mixing and might more directly lead to the commercial application of these materials. At present only one example exists: a multilayer coated contact lens (Excellence™ by CIBA-Vision).

2.2 Molecular recognition

In order to discuss the literature on the use of molecular recognition for materials design, the range to which this term still applies needs first to be defined. In its most pure form molecular recognition can be described as an exclusive binding between two molecules, in which only the combination of these two will result in a high affinity binding event; a combination with any other molecule will not result in binding. This perfect form of molecular recognition does not exist, not even in nature. In example, the well know streptavidin-biotin complex exhibits a very high binding constant driven by multiple hydrogen bonds inside a pocket between biotin and the streptavidin protein, however 2-(4’-hydroxyphenylazo)benzoic acid (HABA) can also bind, although less strongly, to this streptavidin pocket via shape similarities and hydrophobic interactions. This illustrates that the degree of binding strength is often determined by a combination of several interactions (e.g. hydrogen bonding, van der Waals interactions and electronic interactions), however just size resemblance between the ligand and the accepting pocket can still facilitate binding to some degree. The “lock-and-key” mechanism between proteins and ligands, as it frequently found in
nature, can therefore be inhibited by other chemically related molecules that bind to certain, but not all, structural features of the binding site. If the molecular recognition between the protein and the corresponding ligand was perfect, this competition effect between resembling molecules and the corresponding ligand would not be observed. As a result many chemical compounds would not be toxic, however also many medicines would not work, since both are often based on competition and blockage of protein binding sites. The term “molecular recognition” therefore is extremely broad and the specificity, which is in fact the degree of binding strength of a particular molecule compared to a competitor, determines whether the interaction can indeed be classified as molecular recognition. Following this definition, \( \pi-\pi \) interactions between aromatic systems can be classified as being part of molecular recognition when compared to non-aromatic molecules. For example, the aromatic dye ethydium bromide (EtBr) intercalates between the aromatic stacks of the bases within a DNA molecule. Because non-aromatic dyes do not, this might therefore be classified as a molecular recognition interaction between DNA and EtBr. However, the specificity of this recognition is rather limited since many aromatic structures are capable of intercalating with DNA. Introduction of additional interactions increases the specificity of the recognition process and the term molecular recognition therefore covers a broad area ranging from one type of interaction (e.g. \( \pi-\pi \) overlap) to an array of several cooperative interactions as mentioned above. Since this thesis uses hydrogen bonds of the bis-ureido functionality as the recognition motif, this literature overview will be restricted to molecular recognition based on hydrogen bonds in synthetic systems.

### 2.2.1 Hydrogen bonding driven molecular recognition

Inspired by nature many synthetic systems for molecular recognition using hydrogen bonds have been designed. In particular, the double and triple hydrogen bonding motifs of the DNA base pairs adenine-thymine and guanine-cytosine, respectively, have been explored extensively. These interactions are based on arrays of donor (D) hydrogen and acceptor (A) oxygen or nitrogen atoms. Needless to say, larger arrays consisting of many D-A pairs result in an increased association constant and a higher specificity in molecular recognition. Since hydrogen bonds are mainly based on electrostatic induction resulting in a polarization of the donor (positive charge) and the acceptor (negative charge), the binding strength (measured by the association constant) is dependent on electron withdrawing and donating substituents near the hydrogen bonding motive, which can therefore be employed to tune and regulate the specificity and strength of the system. Many studies on a variety of different D-A arrays have
been performed, in which the association constants have been determined as a function of the electron withdrawing or donating nature of substituents.\textsuperscript{95} For triple hydrogen bonded systems AAD-DDA and DAD-ADA arrays are known, however due to the chemical nature of most base pair inspired systems one motive can change into another via tautomerization, or more than one binding motive is possible within the same system, both resulting in a reduced specificity in molecular recognition. For example, the ureidopyrimidone system can dimerise via the ADAD-DADA motif (4), but also via the tautomeric form DDAA-AADD (3) of which the latter is the most favorable due to more attractive and less repulsive secondary interactions between neighboring donor and acceptor groups.\textsuperscript{96-98} The ability to form hydrogen bonding motifs at more than one site is also observed in nature, in which the “normal” Watson-Crick (W-C) base pairing results from the anti-anti combination, however, the interaction between the anti and syn conformation leads to Hoogsteen base pairing, which is only slightly less favorable than the W-C orientation.\textsuperscript{99,100} To improve the specificity and to increase binding strength many variations have been developed and investigated ranging from two-fold\textsuperscript{101,102} to even six-fold\textsuperscript{103,104} hydrogen bonded systems and supramolecular architectures (Figure 5B).\textsuperscript{105,106} Amongst these systems roughly three types can be distinguished: barbiturates (BAR), the ureidopyrimidinone (Upy) derivatives, and the urea-based functionalities (Figure 4).

The application of barbiturates (barbituric acid derivatives) within the concept of molecular recognition led to the development by the group of Hamilton of 2,6-bis(diamidopyridine)-phenol (1) derivatives that were able to recognize the BAR (2) using six-fold hydrogen bonds.\textsuperscript{107} Lehn and coworkers utilized this concept to construct supramolecular polymeric materials.\textsuperscript{108,109} BARs can also be selectively recognized by triaminotriazine (TAZ) derivatives via two triple hydrogen bonding motives and corresponding studies of this system at the air-water interface have been reported frequently.\textsuperscript{110-114} The Upy functionality has been mainly developed and studied within the Eindhoven laboratory and started with the development of the ureido-s-triazine unit, which was able to dimerize via a linear array of quadruple hydrogen bonds.\textsuperscript{115} Placed on the end of short polymer segments, these units were able to induce the formation of a supramolecular polymer, which was able to form a helical stack.\textsuperscript{116-118} The moderate dimerization constant (\textasciitilde10\textsuperscript{3}) led to further development of the Upy unit (3), which also used quadruple hydrogen bonds to achieve a 1000 fold higher dimerization constant.\textsuperscript{119-121} Zimmerman and coworkers developed a system which closely resembles the Upy unit achieving a dimerization constant of \textasciitilde10\textsuperscript{8}.\textsuperscript{122} To improve on the selectivity of both Upy-based systems, 2,7-diamido-1,8-naphthyridine (Napy)(5) was introduced, which, being only of the DAAD type, is not able to dimerize. The Napy unit does bind selectively to the tautomeric ADDA form of the Upy (Figure 5A).\textsuperscript{123-125} Although other
variations have been reported,\textsuperscript{126-128} materials have mainly been constructed using the Upy-polymers.\textsuperscript{129}

![Chemical structures](image)

**Figure 4.** Selected hydrogen bond driven molecular recognition units, with dashed lines indicating the hydrogen bonds between donor (D) and acceptor (A). Six-fold hydrogen bonded barbiturate system (1-2); Upy quadruple hydrogen bonded dimers in both tautomeric DDAA (3) and ADAD (4) forms; Upy-Napy four-fold hydrogen bonded recognition (5); Bis-ureido unit with even (blue) (6) and odd (red) (7) spacer and non-matching a-selective binding between the two types (8).

The final molecular recognition motive is the urea functionality, which is able to form arrays of urea moieties using bifurcated hydrogen bonds.\textsuperscript{130} The combination of several urea groups separated by a spacer results in the formation of bis-\textsuperscript{131,132} and tris-urea\textsuperscript{133} units, which due to their strong binding properties mostly result in insoluble molecules when more than two urea units are positioned within close proximity of each other. Since the use of a single urea unit only leads to moderate selectivity in molecular recognition,\textsuperscript{134} most urea based systems are of the bis-urea type. Because the hydrogen bonds are directional an odd-even effect emerges from the spacer length between two urea functionalities: urea units with an even spacer are oppositely oriented (Figure 4, 6), whereas units with an odd spacer are both oriented
in the same direction (Figure 4, 7). This effect results in an additional selectivity in molecular recognition since two bis-urea units can only stack perfectly if their spacer lengths matches (Figure 4, 8).

Figure 5. A. Upy-Upy and Upy-Napy based supramolecular polymers.123 B. Supramolecular assembly between a barbituric acid- and a triaminotriazine derivative.105 C. Bis-urea based poly(THF) thermoplastic elastomers; inset showing AFM phase image of fiber-like bis-urea hard block segments in a soft polymer matrix.135 D. Bis-urea based organogeleator, with stacking of the bis-urea units inducing a fiber-like morphology (TEM image, Pt shadowing, scale-bar: 500 nm) capable of gellating a multitude of organic solvents.136

This stacking often results in fiber like structures,135,137-141 in which the stacking direction can be recognized. For example, Versteegen showed that the incorporation of a bis-urea unit into the polymeric structure of poly(THF) leads to the formation of fiber-like structures in the phase image of the AFM. This was ascribed to the presence of bis-urea stacks that formed hard-block section in a soft THF polymer matrix (Figure 5C). The resulting material is highly elastic with a high strain at break due to the presence of these hard-block segments. In addition, since at high temperatures the hydrogen bonds between the urea units dissociate, the polymer flows at these conditions and can be processed like a thermoplastic material, which upon cooling
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regains its elastomeric properties.\textsuperscript{135,141} Due to the tendency of bis-urea based molecules to form fiber-like structures, many are capable of forming both organo-\textsuperscript{132,133,136,142-146} (Figure 5D) and hydrogels.\textsuperscript{147}

### 2.2.2 Introducing functionality through molecular recognition

The utilization of molecular recognition via hydrogen bonds to anchor functionality into different supramolecular assemblies and polymers using a modular approach has been demonstrated in only a limited number of cases. In this approach different functional guest molecules are equipped with a molecular recognition site, which upon mixing with the host material will result in recognition and anchoring into the structure. The selectivity of the process can be demonstrated by using guest molecules equipped with a non-matching recognition site, resulting in the absence of guest functionality in the final construct. From the above mentioned types, this has not been demonstrated yet for the BAR system. An example of selective functionalization using hydrogen bond driven molecular recognition via a modular approach has been reported for the Upy-system by Dankers.\textsuperscript{148} It was demonstrated that short peptide fragments, fitted with a Upy functionality, upon mixing with a Upy-based supramolecular polymer could be incorporated into the polymer material resulting in an end product which displayed the functionality of the peptide (Figure 6). Because most supramolecular polymers also use the recognition site (situated at both polymer chain ends) to form high molecular weight chains, guest molecules with the same recognition unit can act as chain stoppers resulting in the disruption of the supramolecular polymer and a lowering of the molecular weight of the resulting material accompanied by poorer material properties. The amount of functional guest that can be added is therefore limited. Arrays of bis-urea units are better suited for this application, since the hydrogen bonds are directed perpendicular to the molecular backbone and insertion of a matching bis-urea anchor of the guest molecule does not result in disruption of the urea stacks. Koevoets et al. demonstrated that thermoplastic elastomers composed of bis-urea functionalized poly(THF) were able to incorporate matching bis-urea guest molecules within the hard block urea sections (Figure 7A).\textsuperscript{149} The selectivity of the molecular recognition was demonstrated using bis-urea functionalized dye molecules equipped with either a matching or a non-matching spacer between the urea units. It was shown using extraction with a detergent solution that the matching dyes were more effectively bound into the polymer material. Wisse et al. demonstrated a similar effect for bis-urea based poly(\(\varepsilon\)-caprolactone) thermoplastic elastomers.\textsuperscript{150,151}
Figure 6. Upy-functionalization of two short peptide sequences (GRGDS and PHSRN) and incorporation into a supramolecular PCL polymer.¹⁴⁸

Figure 7. A. Electro spun (ES) bis-urea based poly(THF) with incorporated matching and non-matching dyes. SEM images show the obtained ES fibers; graph displays the released percentage of dye showing a significant larger retention for the matching dye.¹⁴⁹ B. Cryo-TEM image of micellar triblock copolymer rods. Fluorescence spectra of pyrene bis-urea functionalized guest in CHCl₃/15% TFA incorporated in bis-urea triclock copolymer micellar rods 1 and incorporated in similar block copolymers without a bis-urea unit (2).¹⁵² C. Model for the incorporation of chiral R,R-azobenzene guest in an aggregate of R,R bis-urea based host (top) and in an aggregate of a host of opposite S,S configuration.¹⁴²
Moreover, it was demonstrated that cylindrical micelles of bis-urea based amphiphilic ABA tri-block copolymers could also be functionalized with a guest composed of a matching bis-urea containing center A-block that was fitted with two pyrene moieties at the position of the B-blocks. It was demonstrated by Chebotareva et al. that the excimer fluorescence of the pyrene unit was completely absent when mixed with the bis-urea based blockcopolymer, which is indicative of spatial separation between the pyrene groups during the lifetime of the excited state (Figure 7B). Incorporation of the pyrene guest in a similar ABA tri-block copolymer without bis-urea functionality resulted in the clear presence of excimer fluorescence.\textsuperscript{1522} De Loos et al. even showed chiral recognition of azobenzene modified guests within organogelator hosts; both molecules were bis-urea based and contained two stereogenetic centers (Figure 7C).\textsuperscript{1533} The molecular recognition specificity with respect to the spacer length between bis-urea units was investigated by De Feyter using STM.\textsuperscript{1544} Finally, although not based on urea units, however still on hydrogen bonds, Jung demonstrated the incorporation of a glucopyranoside into sugar based gelators resulting in a double-helical fibers that could be accurately transcribed into silica using the sol-gel polymerization of tetraethoxysilane.\textsuperscript{1555}

\textbf{2.3 Concluding remarks}

The Layer-by-Layer film deposition technique is a versatile coating technique highly suited for porous and non-planar scaffolds as can be found in the biomaterial field. The use of the LbL films as biomaterial coatings has therefore been proposed by many authors, however, systematic research using \textit{in-vitro} and even \textit{in-vivo} studies have only recently begun. Nevertheless, a large number of papers have already been generated during this short period illustrating the great potential of this technique to the biomaterial and medical field and many more applications are sure to come. From a chemical point of view, debate is still ongoing on the exact structure of the obtained films. Although most biological assays presented here show promising results, it is difficult to relate them to the coating components if the exact surface structure is not known. Most polymer based systems are of a mixed composition due to diffusion of the individual components during layer build up. This mixing additionally hampers the selective incorporation of drugs in predetermined layers. In order to solve the above problems, diffusion needs to be limited which imposes constrains on the versatility of the system. This however, does not entail that diffusion should be completely eliminated, since controlled sustained release relies on diffusion. Nor does it entail that the individual layers should always be separated on a nanometer scale, since a drug release compartment of such a single layer would have only limited capacity. It will therefore be the application which will
determine the final separation and diffusion of the LbL film. Large drug releasing compartments require a diffuse exponential growing system, whereas surface signaling and timed release require perfect separation. Specifically tailored films might be constructed by combining the two systems. Although enough examples of diffuse systems exist, no LbL films layered on a nanometer scale have been reported. The aim of this thesis was to explore this area.

Molecular recognition may be used as a tool to limit diffusion of bioactive functionalities within predetermined layers. The combination of supramolecular polymers or assemblies, equipped with different recognition sites as described above, with the LbL technique can be a powerful approach creating the ability to incorporate functionality with a high selectivity within different layers. The different association constants of the Upy-derivatives can be employed to tune the release rate, whereas variation in spacer length between bis-urea units might be very suited to control the selectivity.

It can be foreseen that the combination of these different fields of biomaterials, coating technology and supramolecular chemistry will allow the development of tailored materials for different biomedical applications.

2.4 References


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An introduction to DNA-based coatings.

Spin coated and polymerizable Langmuir films

Abstract

In this chapter initial experiments leading to the development of Layer-by-Layer DNA-based biomaterial coatings are presented. At first DNA-cationic surfactant complexes dissolved in organic solvent were spin coated on glass substrates to obtain a stable DNA-based coating. However, these coatings proved to be inhomogeneous and could easily detach from the substrate surface when exposed to a culture medium. To improve on the stability, polymerizable cationic surfactants were explored in combination with the horizontal Langmuir-Schaefer transfer of polymerized DNA-surfactant films. Also this approach did not yield the desired coating structure and the obtained results could not be explained at the time. Nevertheless, they inspired the work presented in chapter 7, which eventually provided some insight into the limitations of the above method based on a detailed TEM study.
3.1 Introduction

The use of DNA as a functional biomaterial, instead of its use as a carrier of genetic information, has only recently been suggested. The application of DNA as such a biomaterial is impeded by its nucleolytic degradation and solubility in aqueous body fluids. In order to solve these problems, pioneering efforts have resulted in the fabrication of nucleic acid-containing bulk (bio)material as a self-standing film, which appeared to cause no adverse reactions upon subcutaneous implantation in the back of rats. The self-standing films were formed by mixing an aqueous DNA solution with aqueous solutions of different cationic surfactants resulting in the precipitation of the respective DNA-surfactant complexes. These complexes as reported by Okahata et al. proved to be soluble in organic solvents and casting from chloroform/ethanol solution yielded the self-standing films. DNA-surfactant complexes were prepared using a variety of cationic surfactants, composed of protonated amino acids (glycine, alanine or glutamic acid) of which the acid moieties were coupled to n-alkyl alcohols of various chain lengths (8-12 carbon atoms). The resulting DNA-surfactant complexes possessed antibacterial properties and recently also antifungal properties were demonstrated. In our first approach we aimed at the preparation of biomaterial coatings using spin coated films of these DNA-surfactant complexes. The first part of this chapter will discuss our findings concerning this approach. It is shown that in our study, most of the DNA-cationic surfactant complexes proved to be poorly soluble in organic solvents and the resulting inhomogeneous spin coated films easily detached from the substrate surface in both water and in tissue culture. This prompted us to explore the possibility of using polymerizable cationic surfactants in order to improve the stability of the coating. Since most formed DNA-surfactant complexes, in our assay, proved insoluble in organic solvents, the use of spin coating as a coating technique was reconsidered.

The second part of this chapter discusses the application of Langmuir monolayers of polymerizable cationic surfactants formed on a DNA containing subphase, as possible candidates for the construction of DNA-based biomaterial coatings. The formation of cationic surfactant monolayer films on a DNA containing subphase has been reported frequently in connection with their use as model systems for gene-transfection studies. These films are thought to consist of negatively charged DNA molecules bound directly underneath a closely packed surface of cationic surfactant head groups via electrostatic interactions. Okahata et al. showed that these films can be transferred to a substrate, which resulted in alignment of the DNA fibers upon (vertical) Langmuir-Blodgett (LB) deposition. In contrast, (horizontal) Langmuir-Schaefer (LS) transfer did not yield alignment of the DNA. These transferred DNA-
An introduction to DNA-based coatings. Spin coated and polymerizable Langmuir films

Surfactant films would consist of a layer of surfactant molecules attached to the substrate via the alkyl tails and covered with a layer of DNA interacting with the surfactant head groups. This arrangement seems suited for the application as a DNA-based biomaterial coating, as it would ensure that only the top DNA layer is in contact with the biological environment. To improve the stability of the DNA-surfactant film during transfer but also in the ionic media involved in cell culture experiments, a polymerizable cationic surfactant molecule was designed. It involves a lysine hexadecyl ester of which the ε-amine group is protonated, while the α-amino group is coupled to a polymerizable methacrylate group.

3.2 Spin coated DNA-based films

Tabel 1. Surfactants employed for the preparation of the DNA-surfactant complexes.

<table>
<thead>
<tr>
<th>Gly: R₁ = H</th>
<th>Ala: R₁ = CH₃</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>+H₃N-R₂</td>
<td>C₈H₁₇</td>
<td>C₈H₁₇</td>
</tr>
<tr>
<td>+H₃N-C₁₈</td>
<td>C₈H₁₇</td>
<td>C₁₀H₂₁</td>
</tr>
</tbody>
</table>

DNA-cationic surfactant complexes were obtained by mixing aqueous solutions of DNA and a cationic surfactant with approximately equimolar anion-cation ratios. The precipitated water insoluble DNA-surfactant complexes were dissolved in chloroform/ethanol (4:1. v/v) mixtures and spin coated onto glass substrates. A variety of cationic surfactants (Table 1) was used, however most complexes suffered from solubility problems even in chloroform/ethanol mixtures. Consequently, the resulting spin coated films showed an inhomogeneous surface coverage with many micrometer sized deposits on the surface of the glass plate. The DNA-surfactant coating based on the cationic amphiphile glycine-C18 is depicted as a representative illustration (Figure 2A/B). Fluorescent staining of the coating using the DNA intercalating dye Picogreen™ resulted in randomly distributed fluorescent areas, confirming the inhomogeneous distribution of the DNA.
Moreover, exposure to tissue culture medium resulted in a significant decrease in film surface coverage after 3 days and even in complete loss of the coating after 7 days, as was evident from the absence of green fluorescence upon Picogreen™ staining (Figure 2C-F).

Contact angle measurements with water showed that the DNA-surfactant coatings were hydrophobic, with contact angles ~90°, indicating that the alkyl tails of the surfactant molecules are dominant on the surface. However, a rapid decrease in contact angle to ~55° was observed within the time span of 3 minutes, confirming the decomposition of the film. Indeed, contact angles of 50-60 degrees were observed for films that had been exposed to aqueous ionic media (physiological salt solution). Considering the solubility problems of the DNA-surfactant complexes and the inhomogeneity and instability of the resulting films, it was decided to abandon the spin coated DNA-surfactant coatings.

3.3 Polymerizable surfactant-DNA Langmuir monolayers

As the spin coated DNA-surfactant complexes did not yield the desired homogenous DNA-based coatings, a different coating method had to be developed. Transfer of DNA-cationic surfactant Langmuir monolayers should result in a film of closely packed surfactant
molecules attached to the substrate surface on one side and a layer of densely packed DNA on top of it. This coating method would ensure that DNA forms the outer layer of the coating structure. To further stabilize the cationic Langmuir monolayer upon transfer, a polymerizable surfactant was designed and synthesized.

3.3.1 Synthesis

![Scheme 1](image_url)

Scheme 1. Synthesis scheme of the polymerizable surfactant 4.

The design was based on lysine hexadecyl ester of which the alpha and epsilon amine functionalities were used for coupling to a polymerizable methacrylate functionality and for protonation to give a positively charged head group, respectively (Scheme 1). Intermediate 1 was synthesized via a DCC coupling of \(N-\alpha-(Z)-lys-N-\varepsilon-(Boc)-OH\) with hexadecanol. After column chromatography, the Z-group could be selectively and quantitatively removed by catalytic hydrogenation (Pd/C) yielding 2. Reaction of 2 with 2-isocyanatoethyl methacrylate resulted in the formation of 3, which was purified by column chromatography. Finally Boc-deprotection using TFA and subsequent freeze-drying from glacial acetic acid yielded the desired polymerizable surfactant 4.
3.3.2 Langmuir monolayers

Figure 3. Π-A isotherms of 20 μl of surfactant 4 (1 mg/ml) spread and compressed on a water subphase (——) and 5 μl spread on a DNA subphase (3 mg/ml) (---). Arrows show the areas obtained after the extrapolation to zero pressure.

The surface pressure vs. surface area (Π-A) isotherm was recorded by spreading 20 μl of surfactant 4 from a 1 mg/ml chloroform solution onto a water subphase, followed by constant compression until the collapse point was reached. The lift off area was detected at 55 Å²/molecule (Figure 3) and the collapse of the film was observed at ~16 Å²/molecule with a corresponding surface pressure of 22 mN/m. However, no clear 2-D gas-liquid or liquid-solid phase transitions could be observed and the isotherm showed a gradual increase of the surface pressure typical for a liquid condensed phase (L₂). Extrapolation of the isotherm above 18 Å²/molecule to zero pressure yielded a mean molecular area (MMA) of 35 Å²/molecule, which is small considering the polymerizable side tail present in the molecular structure. Moreover,

* Below this MMA no single monolayer can exist, due to the minimum area necessary to accommodate a single alkyl chain (~18 Å²/molecule).
taking into account the moderate surface pressure at the collapse, it can be speculated that crowding causes instability at high compression resulting in an early collapse.

Spreading 4 on a DNA containing subphase (3 mg/ml) resulted in a different isotherm with a shift of the extrapolated MMA in the liquid condensed phase to 110 Å²/molecule and a shift in lift off area to ~250 Å²/molecule. In addition, also a clear liquid expanded state (L₁) could be distinguished with an extrapolated MMA of 210 Å²/molecule, as well as a phase transition between the L₁ and L₂ phase. Consistent with literature, the Π-A isotherm changed significantly when it was compressed on a DNA containing subphase in stead of on a water subphase.⁸–¹³ As tentatively proposed by some papers in the literature on the subject we also suppose that the observed change is due to the formation and compression of DNA-surfactant complexes.¹⁴–¹⁶

### 3.3.3 Polymerization and transfer

In general, the polymerization of surfactant monolayers is characterized by a rapid drop in surface pressure during the reaction at constant surface area, caused by shrinkage of the Langmuir film due to the formation of covalent bonds, which enforce a more tight packing on the surfactant molecules.¹⁷–²⁶ Consequently, monolayer polymerization at constant surface pressure results in a rapid decrease in surface area during the reaction. The polymerization reaction of the DNA-surfactant monolayer was carried out by first spreading surfactant 4 on a DNA containing subphase (3 mg/ml) and subsequently compressing to a surface pressure of 38 mN/m (Figure 4A, C-area). While maintaining constant pressure the monolayer was irradiated with 254 nm UV-light, which resulted in an immediate and fast decrease in surface area (Figure 4A, P-area). The irradiation was continued until a surface area of 26 Å²/molecule was reached after which the monolayer was allowed to equilibrate for 30 min. After polymerization the monolayer was found to be very stable and no decrease in either surface pressure or surface area was observed.

The polymerized DNA-surfactant layer was horizontally (LS) transferred at constant surface pressure onto both hydrophilic Thermanox™ (PET) cell culture coverslips of various sizes (177 mm², 1452 mm²) (Figure 4A, T-area) and quartz substrates (528 mm²). All showed a transfer efficiency of 100% with a high reproducibility, calculated from the decrease in surface area after each transfer (Figure 4B). The high transfer efficiency to hydrophilic substrates is surprising, given the fact that only the hydrophobic alkyl chains of the surfactant molecules were supposed to be exposed at the air-water interface (Figure 5A). Hence, the result pointed to a role for DNA in the transfer process. This suggestion was enhanced by the observation that
only a ~60% transfer efficiency was reached when the monolayer was formed and polymerized on a pure water subphase. In addition, the monolayer collapsed when the polymerization reaction was performed at constant surface pressure on water, indicating that DNA in the subphase had an additional stabilizing effect. Instead, a constant compression rate (barrier speed 1.9 mm/min) was required to drive the polymerization reaction and to prevent the compression rate from increasing as would be the case if the reaction was performed at constant surface pressure.

When using DMPA (2,2-dimethoxy-2-phenylacetophenone) as an initiator for the polymerization reaction (in stead of free radical polymerization at 254 nm UV), an initiator concentration of >20 mole% was necessary to initiate and propagate the polymerization, indicating the absence of a closed continuous array of polymerizable surfactant molecules.

Figure 4. A. Transfer experiment of a polymerized DNA-surfactant monolayer onto 177 mm² thermanox substrates. Compression of a 20 μl surfactant solution (1 mg/ml) spread on a 3 mg/ml DNA containing subphase to a constant surface pressure of 38 mN/m (C), followed by polymerization to a surface area of 26 Å²/molecule using 254 nm UV-radiation. The subsequent transfer is characterized by a rapid decrease in II, due to the sudden removal of monolayer surface, followed by recuperation to the enforced constant surface pressure of 38 mN/m. B. Diagram of the calculated transferred area with an average of 186 mm², corresponding to a 105% transfer efficiency.

3.3.4. Analysis

Angle resolved XPS analysis of the transferred DNA-surfactant monolayers revealed that phosphorus was present close to the substrate surface and not on top of the film, as was expected for a LS-transferred DNA-surfactant Langmuir film (Figure 5A). Instead, a high
content of carbon-carbon bonds (C-C) was observed when measuring the XPS spectrum at a 60° angle with respect to the substrate normal, probing only the top 5 nm of the film.

When the data were recorded at 0°, allowing detection of elements in the top 10 nm of the film (Figure 5B), the relative C-C signal decreased. Thus, the observed angle dependence for the C-C bonds indicated that the majority of the C-C bonds was located at the coating surface and that the structure of the transferred film was actually the inverse of what would be theoretically expected. The presence of hydrophilic DNA at the substrate surface can explain
the observed high transfer efficiency to hydrophilic substrates. It therefore appears that the generally accepted model of DNA bound directly underneath a surfactant monolayer does not apply to this system. Instead, based on the observed high transfer efficiency to hydrophilic substrates, the presence of DNA close to the substrate surface and the substantial amount of initiator molecules required to propagate the polymerization reaction, it is more likely that a mixed phase of DNA-surfactant complexes has been formed. This phase would consist of DNA and surfactant molecules held together by covalent bonds and ionic interactions (Figure 5C), which displays both hydrophobic and hydrophilic areas at the air water interface resulting in an optimal transfer efficiency even to hydrophilic substrates.

3.4 Conclusion

The formation of DNA-cationic surfactant complexes by precipitation yielded either insoluble matter or, when partly soluble in organic solvents, inhomogeneous films that detached from the surface within 7 days during exposure to culture medium. This coating method was therefore abandoned for the construction of DNA-based biomaterial coatings. Transfer of polymerizable DNA-surfactant Langmuir films resulted in films of undesired configuration, in which DNA was positioned not at the coating surface but at the substrate interface. The reason why DNA is localized close to the substrate rather than on the surface of the transferred film was unclear at this time. However, the formation of DNA-surfactant complexes rather than the common 2-layer system, in which DNA is bound underneath a closed layer of cationic surfactant molecules, could account the observed behavior (see also chapter 7).

Since transfer of the DNA-surfactant monolayer did not yield the desired coating structure with DNA positioned on the outside of the film, it was decided to abandon also this coating method.† Moreover, the transfer of Langmuir films is only applicable to primarily flat substrates, therefore, other dip coating techniques are more suitable for implants that often show a complex shape. In addition, initial experiments using the Layer-by-Layer film

† Although unsuited for the application of DNA-based biomaterial coatings, the precursor molecules of the polymerizable surfactant 4 proved to be successful in inducing chirality in supramolecular perylene stacks.(29) This research was performed in collaboration with and at the Max Planck Institute of Colloids and Interfaces, Potsdam, Germany. Precursors 1 and 2 were Boc-deprotected resulting in surfactants with one cationic head group (5) and two cationic headgroups (6), respectively. Due to the lysine structure, both surfactant molecules are chiral, which resulted in induced bisignate Cotton effects in the circular dichroism (CD) spectra of the perylene dye when 5 and 6 were complexed to negatively charged non-chiral sulfonic acid perylene derivatives. Since this subject lies outside the scope of this thesis a more detailed discussion is given in the article.(28)
deposition technique proved to be more promising as will be shown in the next chapters of this thesis.

3.5 Experimental

**General materials and equipment.** Salmon Sperm DNA (~300 bp/molecule; sodium salt) was kindly provided by Nichiro Corporation (Yokosuka-shi, Kanagawa prefecture, Japan). Potential protein impurities in the DNA were checked using the BCA protein assay (Pierce, Rockford, Illinois, USA) and measured to be below 0.20% w/w. The cationic amino acid based surfactants were kindly provided by Fukushima and Hayakawa (Glycine-C8,10,12,16 and 18, Alanine-C8,10,12,16 and 18, Glutamic acid-2C8,10,12,16,18). The cationic surfactant Gly-C18 was also synthesized in our lab using a standard DCC coupling of octadecanol and N-boc-gly-OH, followed by purification using column chromatography. Protonated n-alkyl amines (NH3·C8,10,12,16 and 18) were obtained by freeze-drying of the corresponding commercial available amines (Sigma-Aldrich) from glacial acetic acid. Cetyltrimethylammonium bromide (CTAB, Sigma), 2-benzylxoycarbonylaminono-6-tert-butoxycarbonylamino-hexanoic acid dicyclohexylamine salt (Z-lys(boc)-OH DCHA, Fluka), hexadecanol (Fluka), N,N-dicyclohexylcarbodiimide (DCC, Fluka), 4-(dimethylamino)pyridine (DMAP, Acros), Pd/C (10% Pd, Merck), trifluoroacetic acid (TFA, Fluka) and 2-isocynatoethyl-methacrylate (Aldrich) were used as received. All solvents were purchased from Acros Chemica or Sigma-Aldrich and were of analytical quality (p.a.). The water used during synthesis was demineralised prior before use. Ultra pure water (Barnstead EASYpure LF system; R>17.7 MΩ-cm) was used for the Langmuir experiments and the preparation of the DNA-surfactant complexes. Langmuir isotherms were recorded on a home-build Teflon trough (200 x 75 mm) mounted on a Trans-Tek transducer (Connecticut USA). The surface pressure was recorded using a filter paper (6 x 8 mm) (the bis-urea surfactant adsors the platinum of the Wilhelmy plate). The polymerization reaction was carried out using a Dr Hönlé Uvaspot 400/T with H/2 filter (0.72 W/cm² intensity) irradiating at 254 nm. XPS measurements were performed using a non-monochromized VG Escalab 200 spectrometer equipped with an aluminum anode (Al Kα; 1486.6 eV) operating at 510 VA with a background pressure of 2 x 10⁻⁶ mbar. Spectra were acquired at 0° and 60° with respect to the surface normal. Contact angle measurements were performed on a Krüss Drop shape analysis system DSA 10 using the sessile drop method. Scanning electron microscopy and fluorescent microscopy experiments were performed at the Radboud University Nijmegen, Departement of Periodontology & Biomaterials.

**Hexadecyl 2(S)-benzylxoycarbonylamino-6-tert-butoxycarbonylamino-hexanoate (1).** Z-lys(boc)-OH was commercially available as a dicyclohexylamine salt. To obtain the free acid form, we suspended Z-lys(boc)-OH DCHA salt in ethyl acetate and washed it with a 5% citric acid solution until both phases were no longer turbid. After separation, the organic phase was washed twice with water and once with brine. Subsequent drying over magnesium sulfate followed by filtration and evaporation of the solvent yielded the free acid form as a yellow oil, which was used for further synthesis. Z-Lys(boc)-OH (2 g, 5.5 mmol, Mw: 366.41) and hexadecanol (1.2 g, 5.0 mmol, Mw: 242.44) were dissolved in dry dichloromethane, and the mixture was cooled to -20 °C. While being stirred vigorously, DCC (1.2 g, 5.8 mmol, Mw: 206.33) and DMAP (70 mg, 0.58 mmol, Mw: 120.68) were added in quick succession, and the mixture was allowed to warm to room temperature after which a white precipitate was formed. The reaction mixture was then stirred for 24 hrs at room temperature. Dicyclohexylurea (DCU) was filtered off and weighed to determine whether the reaction had reached completion. After evaporation of the solvent, the crude product was obtained in 75% yield. The latter was purified by column chromatography on a silica gel column using
15 vol% ethyl acetate in chloroform as the mobile phase. 1H NMR (CDCl3): δ 0.9 (CH3CH2, 3H, t), 1.2-1.9 ((CH2)15CH3, 28H, m and (CH2)2NH, 6H, m), 1.4 (C(CH3)3, 9H, s), 3.1 (CH2NCOOC(CH3)2, 2H, dd), 4.1 (COOCCH2CH2, 2H, t), 4.3 (NHCONHCOO, 1H, dd), 4.5 (NHCOOC(CH3)2, 1H, t), 5.4 (NHCOOC(CH3)2, 2H, s), 7.3 (C6H5, 5H, m). MALDI-TOF-MS: m/e 505 (M+ - boc + 2H), 627 (M+ + Na), 643 (M+ + K).

Hexadecyl 2(S)-amino-6-tert-butoxycarbonylamino-hexanoate (2). For Z-group deprotection, 1 (0.5 g, 0.83 mmol, Mw: 604.86) was dissolved in methanol to which a catalytic amount of Pd/C was added. The system was purged with a stream of nitrogen to remove all oxygen. Hydrogenation was carried out overnight using a shaken type Parr reactor operating at 80 psi hydrogen overpressure. The reaction was monitored by 1H NMR until complete deprotection of the Z-group. The catalyst was removed by filtration over Celite and the solvent, formed toluene and CO2 were evaporated. The crude product was obtained in almost quantitative yield and further purified by column chromatography on a silica gel column using 80 vol% ethyl acetate in chloroform as the mobile phase. 1H NMR (CDCl3): δ 0.9 (CH3CH2, 3H, t), 1.2-1.9 ((CH2)15CH3, 28H, m and (CH2)2NH, 6H, m), 1.4 (C(CH3)3, 9H, s), 1.8 (CH2NH2, 2H, m), 3.1 (CH2NCOOC(CH3)2, 2H, dd), 3.5 (CHCOOC(CH3)2=CH2, 1H, m), 4.1 (COOCCH2CH2, 2H, t), 4.5 (NHCOOC(CH3)2, 1H, t), MALDI-TOF-MS: m/e 415 (M+ - C(CH3)3 + H), 471 (M+ + H), 493 (M+ + Na).

Hexadecyl 2(S)-l2(methacryloyloxy)-ethylaminocarbonylamino-6-amino-hexanoate trifluoroacetic acid (4). Compound 2 (320 mg, 0.68 mmol, Mw: 470.73) was dissolved in dichloromethane, after which 232 mg (1.5 mmol, Mw: 155.15) of 2-isocyanatoethyl methacrylate was added and stirred for 24 hrs at room temperature. The reaction mixture was evaporated under high vacuum to remove the solvent and traces of unreacted isocyanate yielding 3, which was subsequently purified by column chromatography using 30 vol% ethyl acetate in chloroform as the mobile phase. After purification the Boc-group was removed by dissolution of 3 in 90 vol% TFA/water, which was left stirring for an additional 30 min. Polymerizable surfactant 4 (120 mg, 0.19 mmol, Mw: 639.79) was obtained in 28% yield via subsequent evaporation of TFA followed by freeze drying from glacial acetic acid. The low yield can be explained by a tedious work-up procedure that was not optimized. 1H NMR (CDCl3): δ 0.9 (CH3CH2, 3H, t), 1.2-1.9 ((CH2)15CH3, 28H, m and (CH2)2NH, 6H, m), 1.9 (COOC(CH3)2=CH2, 3H, s), 3.0 (CH2NH2, 2H, m), 3.5 (NHCONHCH2, 2H, dt), 4.1 (CH2CH2OCOCH2, 2H, t), 4.2 (CH2COOC(CH3)2=CH2, 2H, t) 4.3 (COCHNH, 1H, dd), 5.6 (cis-C(CH3)2=CH2, 1H, s), 6.1 (trans-C(CH3)2=CH2, 1H, s), 8.0 (NH, 3H, m). MALDI-TOF-MS: m/e 526.44 (M+), 548.38 (M+ - H + Na), 564.35 (M+ - H + K). Anal. calcld. (%) for C31H48F6N2O: C 58.2, H 8.8, N 6.6; Found (%): C 57.6, H 8.8, N 6.5.

Spin coated DNA-surfactant coating preparation. DNA-cationic surfactant complexes were prepared by mixing an aqueous DNA solution (2 mg/ml) and an aqueous cationic surfactant solution (3 mg/ml), both dissolved in Milli-Q water at a DNA:surfactant ratio of 1 to 1.1. The instantly formed precipitate was collected via centrifugation and washed 3 times with ultrapure water. The obtained DNA-surfactant complexes were freeze dried after the final washing step. Many of the obtained complexes were insoluble in all solvents, however, some appeared to be fairly well dissolved/dispersed after sonication in chloroform/methanol mixtures (4:1). The resulting DNA-surfactant complex solutions in organic solvent were spin coated onto glass culture dishes and analysed using SEM, contact angle measurements and picogreen staining. The employed cationic surfactants are mentioned in the general materials section.

Langmuir experiments. A 1 mg/ml chloroform solution of the polymerizable surfactant 4 was prepared. In general 20 μl was spread (unless indicated otherwise) and compressed (barrier speed: 1.9 mm/min) after an equilibration period of 30 min. The subphase was either ultrapure water or a DNA solution (3 mg/ml) prepared using ultrapure
water. To record the Π-A isotherm of surfactant 4 on a DNA subphase starting from Π = 0 mN/m, 5 μL of 4 (1 mg/ml) was spread.

**Polymerization and transfer.** Surfactant 4 (20 μl, 1 mg/ml) was spread on a DNA subphase (3 mg/ml), after which the system was compressed to and maintained at a surface pressure of 38 mN/m. At constant pressure, the UV-lamp, which was positioned 30 cm above the trough, was switched on until a MMA of 26 Å²/molecule was reached (5 min. irradiation), after which the system was left 30 min. to equilibrate. While still maintaining constant pressure the DNA-surfactant monolayer was transferred to Thermax™ or quartz substrates using horizontal (LS) transfer by touching the surface of the air water interface with a horizontally positioned substrate. Excess water was carefully removed using a filter paper positioned at the side of the substrate. It should be noted that the Π-A isotherm did not start from 0 mN/m surface pressure, due to the larger amount of spread surfactant necessary to reach Π = 38 mN/m and leave enough surface area to transfer the monolayer after the additional compression caused by the polymerization reaction. As a result the isotherm showed a steeper liquid condensed phase with a smaller extrapolated MMA (70 Å²/molecule) when compared to the isotherm that started at 0 mN/m. Additionally a second phase transition following the liquid condensed state could be observed at a MMA of 38 Å²/molecule.

The polymerization of 4 on a water subphase was performed at a constant compression rate (1.9 mm/min.) after a surface pressure of 12 mN/m was reached. The surfactant spread on a water subphase could not be compressed to a high surface pressure of 38 mN/m, therefore a lower surface pressure was chosen. In addition, due to the steep incline of the isotherm, compression to higher surface pressures would not have left enough surface area to accommodate the additional compression caused by the polymerization reaction and subsequent transfer of the monolayer. The monolayer was irradiated for 5 min. resulting in a decrease in surface pressure due to the slower compression rate when compared to the polymerization at constant pressure. Following the polymerization reaction the system was compressed back to and maintained at a surface pressure of 12 mN/m. and left to equilibrate for 30 min., after which the monolayer was LS transferred to Thermax™ substrates as described above.

For the polymerization using the DMPA initiator several surfactant solutions were prepared in chloroform with a 1 mg/ml concentration of 4, to which different amounts of DMPA ranging from 4 to 20 mole% were added. The polymerization was performed in a similar fashion as described above only with a UV-filter (pass >365 nm).

**X-ray photoelectron spectroscopy (XPS).** XPS-Spectra were acquired at 0° and 60° with respect to the surface normal of the LS transferred polymerized DNA-surfactant film deposited on Thermax™ substrates. Peak intensities were normalized on the phosphorus signal.
3.6 References


Polymer-based Layer-by-Layer coatings – Analysis of a mixed system

Abstract

The previous chapter revealed that both spin coating of DNA-surfactant aggregates and the Langmuir-Schaefer transfer of polymerized DNA-surfactant monolayers could not be used to construct an applicable DNA-based biomaterial coating. As shown in this chapter, our search for a suitable coating method led us to the use the Layer-by-Layer deposition technique, in which the anionic DNA and cationic polyelectrolytes are absorbed in an alternate fashion onto a substrate by dipping from an aqueous solution. The films which are based on poly-D-lysine and poly(allylamine hydrochloride) as cationic components were chemically and biologically analyzed. It was demonstrated that the surfaces of both coatings had a mixed composition of DNA and the cationic polyelectrolyte. Biological assays showed promising results with respect to the future application of the films as biomaterial coating.
Chapter 4

4.1 Introduction

In the previous chapter it was shown that DNA-surfactant complexes spin-coated from organic solvents yielded irreproducible and inhomogeneous films, rendering this route unsuitable for the preparation of biomaterial coatings. Similarly, the Langmuir-Schaefer transfer of polymerized DNA-surfactant monolayers, although readily applied to flat substrates, is not a suitable coating method when dealing with 3-D or even porous objects. It was therefore decided to switch to the more convenient and more universal Layer-by-Layer (LbL) coating technique.

Since its introduction by Decher,\textsuperscript{1–2} this self-assembly technique has received a great deal of attention as a versatile and simple coating method that does not require sophisticated machinery and can be performed using a large range of solvents. It involves the alternate absorption of two mutually attracting components theoretically resulting in a layered structure. When the LbL deposition is performed using electrostatic interactions between oppositely charged polyelectrolytes, it is also referred to as the electrostatic self-assembly technique (ESA). In principle, due to their layered structure, LbL films can act as a multi-compartment drug delivery material, making them ideal candidates for use in the field of biomaterial coatings. The literature overview in chapter 2 indeed shows many examples of the application of the LbL technique in the biomaterial field.

DNA, being an anionic polyelectrolyte, has already been applied successfully in conjunction with the ESA technique for the construction of polyelectrolyte multilayers (PEMs) using cationic polyelectrolytes as positively charged counterparts.\textsuperscript{3–14} Interestingly, the electrostatic interactions with positively charged polyelectrolytes have been demonstrated to protect DNA from degradation by nucleolytic activity.\textsuperscript{15} Even immersion of PEMs in solutions of high ionic strength (i.e. a strength exceeding those in physiological conditions) does not cause dissociation of the PEM structure. Instead, varying the ionic content of the polyelectrolyte solutions during the fabrication process is one of the means to modulate PEM properties, including layer thickness\textsuperscript{2,12,16} Although DNA-based LbL films have been reported, the potential application of these films was mostly related to DNA-based sensors or gene transfection and their application as bioactive coating thus far has been unexplored.

In this chapter we report on the preparation and characterization of DNA-based LbL films to be used as a biomaterial coating. Subsequently, we discuss their biological evaluation. Prior to the biological assays, complete chemical characterization of the coating was performed in order to relate any biological response to the structure of the coating. The LbL process is fairly well understood,\textsuperscript{17–21} however reports on the detailed morphological and chemical
characterization of PEMs are scarce. The proposed anti-inflammatory properties of DNA necessitate the quantification of the amount of DNA immobilized in the coating and particularly at the coating surface. PEMs can show either a linear or an exponential growth profile, depending on the polyelectrolytes used.\textsuperscript{17,18,20-24} Therefore, two different polymers were selected as cationic polyelectrolyte component in the DNA-based LbL films: poly-D-lysine (PDL) from the exponential\textsuperscript{12} and poly(allylamine hydrochloride) (PAH) from the linear growing family.\textsuperscript{11} The obtained PEMs were characterized using UV-Vis spectroscopy, radiolabeling, X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM) and Fourier transform infrared spectroscopy (FTIR).

The biological evaluation comprised the study of \textit{in vitro} cytological as well as \textit{in vivo} histological response, \textit{in vitro} inflammatory response using macrophage models, the deposition of calcium phosphates and the behavior of bone-forming cells (osteoblasts) on the films. In addition, we evaluated the possibilities of functionalizing these coatings with additives, e.g. the osteoinductive’ growth factor: bone morphogenetic protein 2 (BMP-2). All biological assays were performed in collaboration with the Radboud University Nijmegen, Department of Periodontology & Biomaterials.

\subsection*{4.2 Coating construction}

LbL multilayer films were deposited on titanium, glass and quartz substrates, as described by Luo et al.\textsuperscript{5} with a few modifications. Cleaned substrates were immersed in an aqueous solution of either PDL (0.1 mg/ml) or PAH (1 mg/ml) for 30 minutes, allowing sufficient time for the adsorption of the first cationic polyelectrolyte layer, since the complete deposition of the first layer is important for an effective build-up of the structure. Subsequently, the substrates were washed in ultra-pure water for 5 minutes and dried using a pressurized air stream. Thereafter, they were alternately immersed in an anionic aqueous DNA solution (1 mg/ml) and the respective cationic polyelectrolyte solution for 7 minutes each, with intermediate wash (5 min) and drying steps. The build-up of the multilayered DNA-coatings was continued until a total number of 5 double-layers was reached. These were designated either [PDL/DNA]$_5$ or [PAH/DNA]$_5$.\textsuperscript{1}

\footnote{Promotes bone formation.}

\footnote{The denotation of coatings is restricted to indicating the number of double-layers, i.e. $\frac{1}{2}$ represents only the cationic part of the double-layer.}
Figure 1. A. UV-vis absorption spectra of [PAH/DNA]-multilayer coatings on a quartz substrate for different numbers of deposited DNA-terminated double-layers (1-5). B. Absorbance at 260 nm plotted versus the number of double-layers (DNA-terminated) of A showing a linear increase. C. UV-Vis absorption spectra of [PDL/DNA]-multilayer coatings on a quartz substrate for different numbers of deposited DNA-terminated double-layers (1-5). D. Absorbance at 260 nm plotted versus the number of double-layers (DNA-terminated) of C showing an exponential increase.

The build-up of multilayered DNA-coatings on quartz substrates was monitored using the characteristic 260 nm UV-absorption of the nucleic bases. As illustrated in Figure 1, both multilayered DNA-coatings showed an increase in UV-absorbance spectrum with each successively added double-layer. The UV-absorbance of the [PDL/DNA]₅ double-layers was higher than that of the [PAH/DNA]₅ double-layers, indicating a larger amount of DNA deposited on the surface in the former case. In agreement with literature reports, when peak values at 260 nm were plotted against the number of double-layers, a linear increase is...
apparent for the [PAH/DNA]-PEM system (Figure 1B). In contrast, the [PDL/DNA]-PEM system could be fitted better to an exponential curve (Figure 1D).

LbL deposition on titanium substrates using radio-labeled DNA indicated that on average, for both [PDL/DNA] and [PAH/DNA] coatings, 3 μg/cm² of DNA was deposited upon each DNA addition step. It should be noted, however, that the deposition efficiency on titanium substrates can deviate from the efficiency on quartz.

4.3 Coating chemical analysis

4.3.1 XPS

The XPS spectra of drop cast DNA and of 1, 3½, and 5 double-layers of [PDL/DNA] as well as of [PAH/DNA] on titanium-sputtered silicon substrates were recorded both at 0° and 60° with respect to the normal axis. The XPS spectrum of the drop cast DNA showed the presence of magnesium, most probably as a counter ion, whereas almost no sodium or potassium peaks could be detected. The XPS spectra of DNA-terminated [PDL/DNA]-coatings showed the presence of Mg-peaks. In contrast, DNA-terminated [PAH/DNA]-coatings did not show the presence of Mg-peaks, nor did coatings terminated with a cationic PDL or PAH layer (Figure 2). Furthermore, a distinct difference was observed with respect to the presence of titanium (Ti) peaks. Whereas the [PAH/DNA]-coatings showed the presence of Ti-peaks, the corresponding [PDL/DNA]-coatings did not.

The phosphorus/nitrogen ratios (IP2p/IN1s) were determined for all recorded spectra, and used as a direct measure for the presence of DNA. The penetration depth, i.e. the maximum layer thickness from which the electrons generated by the x-ray beam are still able to escape and reach the detector, at 0° is ca. 10 nm. However, the depth at 60° with respect to the normal axis is limited to approximately 5 nm. DNA terminated layers should therefore show a larger IP2p/IN1s ratio for the 60° signal than for the 0° one due to phosphor enrichment in the top 3 nm (DNA chain thickness ~2.5 nm). The PDH and PAH terminated layers on the other hand, should show a lower IP2p/IN1s ratio for the 60° signal compared to the 0° one and thus an angle dependence is expected when the DNA and PDL or PAH layers are separated. It should be noted, however, that although an angle dependence for phosphorus indicates the presence of a layered system, its absence does not necessarily point to a completely mixed film. If the thickness of the individual layers is more than the penetration depth of the XPS at 0° or if the surface is extremely rough, no effect will be observed.
Figure 2. A. XPS spectra of a drop cast DNA film and of [PDL/DNA]-multilayers; number of double-layers are indicated. B. Idem as A for [PAH/DNA]-multilayers. The spectra were normalized to a constant integrated intensity of phosphorus 2p emission.

As can be expected for a one-component film, no angle dependency was observed in the \( \text{IP}_{2p}/\text{IN}_{1s} \) ratio in the case of the drop cast DNA film. Interestingly, none of the analyzed [PDL/DNA]- and [PAH/DNA]-coatings showed an angle dependence, implying a homogeneous distribution of DNA in the surface region of the coatings, a thick final layer, or a rough surface. Since the angle dependence was also absent in the initial stages of the build up process of the multilayer, during which the coating is expected to be thin and relatively flat, it is more likely that the absence of an angle dependence is caused by a mixing of the two components in the top of the film. The amount of DNA in the top 5 - 10 nm of the multilayered coatings was calculated using formula (1). From the DNA percentages presented in Table 1 it can be concluded that the surface of [PDL/DNA]-multilayers display a higher DNA content for DNA terminated films compared to PDL terminated ones. It should be noted, however, that with increasing number of multilayers, the percentage of DNA in the DNA terminated layer

\[ \text{IP}_{2p}/\text{IN}_{1s} \]

\[ \text{No increase in } \text{IP}_{2p}/\text{IN}_{1s} \text{ ratio for the DNA-terminated single double layer and no decrease in } \text{IP}_{2p}/\text{IN}_{1s} \text{ ratio for the PAH- or PDL-terminated } 3\frac{1}{2} \text{ double-layer.} \]
becomes lower, which might be the result of a diffusion and mixing process. On the other hand, no alternating effect in DNA content between DNA terminated and PAH terminated layers was observed in the case of the [PAH/DNA]-multilayers. In these films the DNA content increased with increasing number of layers, irrespective of the final deposited component.

\[
\frac{I_{P2p}}{I_{N1s}} = x_{DNA}\left(\frac{I_{P2p}}{I_{N1s-DNA}}\right) \quad (1)
\]

Table 1. Percentage of DNA in the top (5 - 10 nm) layer of coatings deposited on glass as calculated from XPS data using equation (1).

<table>
<thead>
<tr>
<th>Coating architecture</th>
<th>% DNA in [PDL/DNA]</th>
<th>% DNA in [PAH/DNA]</th>
</tr>
</thead>
<tbody>
<tr>
<td>[double-layer]_1</td>
<td>87</td>
<td>52</td>
</tr>
<tr>
<td>[double-layer]_{3½}</td>
<td>66</td>
<td>59</td>
</tr>
<tr>
<td>[double-layer]_3</td>
<td>80</td>
<td>72</td>
</tr>
</tbody>
</table>

XPS spectra of 5 double-layers of [PDL/DNA] and [PAH/DNA] on quartz substrates showed a similar picture: no angle dependence in either of the cases, the [PDL/DNA] film displayed a higher DNA content when compared to [PAH/PDL] and magnesium peaks could be observed only for the [PDL/DNA] multilayer. However, in both cases clear silicon peaks were present in the XPS spectra.

The observed Si- and Ti-peaks belong to the underlying substrate, which indicates either a homogenous thin film < 10 nm or an irregular build-up of the films in domains with dimensions < 10 nm. From these results it can be concluded that both multilayer films are much thicker or more regular on titanium substrates than on quartz. Furthermore, the [PDL/DNA] films on titanium are either thicker or more regular than the [PAH/DNA] films, which is in line with the higher 260 nm UV-absorption observed for the former films. The increased \(I_{P2p}/I_{N1s}\) ratio in DNA terminated layers of [PDL/DNA]_x-coatings compared to the PDL terminated layers, indicates a higher DNA concentration in the top layers, suggesting that these coatings have a layered structure. In contrast, the absence of an angle dependence in the \(I_{P2p}/I_{N1s}\) ratios is characteristic for a mixing of the layers. Most probably these coatings have a mixed composition but are enriched in the last deposited component. For [PAH/DNA]_x-coatings no angle dependence nor a clear enrichment of one of the components in the last deposited layer could be detected. This indicates fully mixed coatings, despite the expected linear growth behavior of the system. Moreover, the absence of Mg-peaks in the DNA-terminated [PAH/DNA]_x-coatings, can be explained by an exchange of most DNA counter ions with the positively charged PAH, supporting mixing of these components.
4.3.2 AFM

The morphology of non-coated titanium-sputtered silicon to which \( \frac{1}{2} \), 2, 3\( \frac{1}{2} \) and 5 double-layers of both [PDL/DNA] and [PAH/DNA] had been deposited, was analyzed by AFM with typical scan sizes between 0.5 x 0.5 µm\(^2\) and 5 x 5 µm\(^2\). The surfaces roughness was determined using Root-Mean-Square (RMS) calculations, a denotation based on the standard deviations of the z-value (vertical direction) (Table 2).\(^\dagger\) For both types of coating, the AFM height images showed an optical as well as a RMS increase in surface roughness upon increasing layer thickness (Figure 3). Furthermore, after the deposition of 2 double-layers, RMS-values of [PDL/DNA]-coatings were significantly lower than those of equivalent [PAH/DNA]-coatings. Typical differences in surface morphology between the two types of coatings included the spatial distribution of elevations, as well as their average height (Figure 4). Whereas [PDL/DNA] showed a relatively homogenous morphological appearance with equally-spaced elevations of relatively small height (average 6 nm), [PAH/DNA] showed randomly-distributed elevations of relatively large height (average 14 nm). For the latter the presence of substrate related peaks in the XPS spectrum implies that areas in which the total coating thickness is less than 10 nm are present. In contrast, the observed surface roughness, in tandem with the absence of Ti-peaks in the XPS spectrum, indicates that for the [PDL/DNA]-coating the film is at least 10 nm thick in the lowest regions between the equally spaced elevations.

\(^\dagger\) The forces of the cantilever on the coatings during contact mode scanning were well below the threshold above which damage occurs due to the imaging process, as evidenced by the absence of damage on AFM-images with increasing scan size.
Table 2. Determination of the surface roughness (nm) based on RMS calculations. *Asterisk indicates a significant increase in surface roughness (p<0.05) compared to previous coating.

<table>
<thead>
<tr>
<th>Coating architecture</th>
<th>[PDL/DNA] (nm)</th>
<th>[PAH/DNA] (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Titanium-sputtered silicon)</td>
<td>1.64 ± 0.03</td>
<td>1.64 ± 0.03</td>
</tr>
<tr>
<td>[double-layer]½</td>
<td>1.68 ± 0.27</td>
<td>1.99 ± 0.10</td>
</tr>
<tr>
<td>[double-layer]²</td>
<td>2.40 ± 0.11*</td>
<td>1.96 ± 0.11</td>
</tr>
<tr>
<td>[double-layer]⁵</td>
<td>3.16 ± 0.17*</td>
<td>4.58 ± 0.96*</td>
</tr>
<tr>
<td>[double-layer]⁵</td>
<td>5.49 ± 0.57*</td>
<td>8.32 ± 1.70*</td>
</tr>
</tbody>
</table>

Figure 4. Three-dimensional morphology images of 5 double-layer coatings on titanium-sputtered silicon substrates. A. [PDL/DNA]⁵ B. [PAH/DNA]⁵

4.3.3 FTIR.

DNA exhibits its characteristic bands of base-pairs at 1602 cm⁻¹ (adenine), 1650 cm⁻¹ (thymine), 1684 cm⁻¹ (guanine), and 1481 cm⁻¹ (cytosine).²⁵ IR-vibrations associated with the sugar-phosphate backbone of the DNA double helical structure can be observed in the 1250 – 950 cm⁻¹ vibration region. DNA has characteristic bands related to the stretching of the phosphate groups (νs 1235 cm⁻¹, νas 1088 & 1063 cm⁻¹), and the C-C stretching of the backbone (963 cm⁻¹).²⁵ Both the [PDL/DNA]⁵ and [PAH/DNA]⁵ multilayered coatings showed a νs at 1088 cm⁻¹. A shift of the νas band from 1235 to 1213 cm⁻¹ for the [PDL/DNA]⁵-coating and from 1235 to 1224 cm⁻¹ for [PAH/DNA]⁵-coating was observed (Figure 5). Shifts of the νas band after complexation of DNA with cationic polyelectrolytes or ions have been observed frequently,⁶,⁷,²⁷ and are explained by the interaction of the anionic phosphate groups of the DNA-molecules with their cationic counterparts. Additionally, the relative humidity of multilayered DNA-coatings is an important factor determining the precise location of these bands.⁷
Figure 5. FTIR-spectra of DNA (upper line), [PDL/DNA]₅ (middle line), and [PAH/DNA]₅ (bottom line). Both types of multilayered DNA-coatings were build-up on quartz substrates.

Although shifts in the ν₅₅ band may also reflect a change in conformation of DNA from e.g. the A to the Z-form or the B to the C-form,¹⁰,²⁶ no conclusions related to the exact structure of DNA can be drawn based only on these IR-spectra.

4.4 Biological analysis of the coatings

In the next section a summary of the studies³⁵-³⁹ related to the biological assessment of the coatings produced in close collaboration with the Radboud University Nijmegen, Department of Periodontology & Biomaterials is given. It demonstrates the potential of DNA-based LbL films as a biomaterial coating.
4.4.1 Cell proliferation, viability and morphology of fibroblast cells

The *in vitro* cell response of rat primary dermal fibroblasts (RDF) was evaluated on both glass and titanium substrates coated with [PDL/DNA]₅ and [PAH/DNA]₅ films, via determination of cell proliferation, viability and morphology. RDF cell proliferation was increased on both types of multilayered DNA-coatings, for both glass (not shown) and titanium substrates (Figure 6) compared to the non-coated controls. This result may be explained as follows. The presence of DNA in the outermost layer of both types of multilayered DNA-coatings might directly influence cell behavior or via the adsorption of proteins. Direct contact between DNA and fibroblasts via supplementation of DNA in the culture medium has been demonstrated to decrease proliferation.²⁸ The increased proliferation of RDF cells on multilayered DNA-coatings in this study demonstrates that inhibiting effects on fibroblast proliferation of DNA in solution are reversed after immobilization, indicating a more prominent role for protein adsorption and furthermore confirms the stability of the coating.

![Figure 6. Proliferation of rat dermal fibroblast (RDF) cells on non-coated control substrates (white bars), [PDL/DNA]₅ (grey bars), and [PAH/DNA]₅ (black bars) coatings on titanium substrates. Results are shown as means ± SD of triplicate wells for one representative experiment out of three. (*) Significantly different from non-coated control substrates p < 0.01. (**) Significantly different from non-coated control substrates p < 0.001. (#), Significantly different from [PDL/DNA]₅-coated substrates, p < 0.01.](image)

The viability of the RDF cells on these coatings was assessed using a Live/Dead® assay and a MTT-test.²⁹ For the Live/Dead® assay, the primary RDF cells cultured for 3 days on both types of multilayered DNA-coatings and non-coated controls, showed high numbers of live (green) cells and hardly any dead (red) cells. No apparent difference in the number of dead (red) cells was observed between non-coated control samples and either type of multilayered
DNA-coatings. On the other hand, an apparent higher number of live cells were observed on both types of multilayered DNA-coatings compared to non-coated control samples, which is in line with the observed higher RDF proliferation as reported above.

![Figure 7](image)

**Figure 7.** SEM images showing the morphology of RDF cells after 3 days of cell culture on A. non-coated controls, B. [PDL/DNA] and C. [PAH/DNA] fabricated on titanium substrates. Note the presence of multiple cellular extensions (arrowheads) of the fibroblasts on all types of substrates. Inset shows enlarged image of the extensions.

The mitochondrial activity was measured using a MTT-based assay. The capacity of the mitochondrial enzyme succinate dehydrogenase to reduce MTT is a measure to evaluate the metabolic activity of cells. Neither type of multilayered DNA-coatings decreased mitochondrial redox activity of RDF cells (after 3 days of culture) compared to non-coated controls (p>0.05), confirming the results of the Live/Dead® assay, which indicate no decrease in viability of the cells.
The morphological appearance of the primary fibroblast cells, cultured on both types of multilayered DNA-coatings and on non-coated titanium controls, was evaluated using scanning electron microscopy (SEM)(Figure 7). Although the native roughness of the as-machined titanium substrates does not allow a clear observation of completely spread cells, it becomes apparent from slightly elevated cells that both types of multilayered DNA-coatings do not alter the morphological appearance of the cells compared to non-coated titanium controls. In general, all cells had a typical fibroblast appearance with several (short) cellular extensions.

In short, the above biological assessments demonstrate an increased proliferation of primary fibroblasts induced by the presence of both types of multilayered DNA-coatings, while cell viability and morphology remain unaffected.

4.4.2 Histological evaluation

For the in vivo experiments, cylindrical titanium substrates were coated with 5 double-layer [PDL/DNA] or [PAH/DNA] coatings and implanted subcutaneous in the back of rats. Tissue samples, from which the implants were removed after 4 and 12 weeks, were embedding in paraffin and subsequently sectioned. Based on morphological criteria these specimens demonstrated relatively mature fibrous capsules surrounding all types of implants without inflammatory cells at the interface between implant and surrounding tissue. SEM analysis of the retrieved implant surface demonstrated only limited coverage with proteinaceous deposits that could not be identified as cellular material.

From these experiments it could be concluded that multilayered DNA-coatings did not affect the quality and thickness of the fibrous capsule. Generally, all capsules consisted of fairly mature tissue without any signs of inflammatory cell infiltration, and both capsule quality and thickness were not significantly different between the two implantation periods (4 and 12 weeks)(Figure 8).
Figure 8. Light microscopic images of tissue capsule surrounding titanium control implants (A, D), [PDL/DNA]₅-coated implants (B, E), and [PAH/DNA]₅-coated titanium implants (C, F) after implantation periods of 4 and 12 weeks. The I denotes the position of the implant.

4.4.3 Immune responses

Assays in which the behavior of macrophages is examined are a useful determinant to study the biocompatibility of biomaterials. The use of macrophages for this purpose is based on their role in the foreign body response. By virtue of their capacity to secrete a large array of products, including soluble mediators (cytokines) and growth factors, macrophages orchestrate the activation of numerous cell types, and are considered to be key regulators of the intensity and duration of the foreign body and inflammatory response. Permanent activation of macrophages during the foreign body response may result in persistent inflammatory reactions, and eventually in the fusion of macrophages into so called ‘foreign body giant cells’ (FBGCs). Two macrophage cell lines (J774A.1 and RAW264.7) were cultured on both types of multilayered DNA-coatings ([PDL/DNA]₅ and [PAH/DNA]₅) on glass for 24 and 48 hours. Non-coated glass substrates served as negative controls, whereas stimulation with
lipopolysaccharide (LPS) was used as the positive control. Macrophage behavior was assessed in terms of proliferation, viability, morphology, and cytokine secretion of tumor necrosis factor alpha (TNF-α), transforming growth factor beta1 (TGF-β1), interleukin 10 and beta1 (IL-10, IL-1β). These cytokines have been classified according to their role in the foreign body response (Figure 9C).\textsuperscript{32,33}

Cell culture experiments showed that the attachment to, and viability and morphology of both types of macrophages cultured on the multilayered DNA-coatings were comparable to non-coated controls. In contrast to RDF cells, macrophages did not show an increase in cell proliferation, indicating that the observed effect for RDF proliferation is cell-type specific. RAW264.7 macrophages cultured on [PDL/DNA]\textsuperscript{5} showed TNF-α secretion levels comparable to the negative controls (p>0.05). On the other hand, RAW264.7 macrophages cultured on [PAH/DNA]\textsuperscript{5} showed a significantly decreased TNF-α secretion after both 24 (p<0.05) and 48 hours (p<0.01) of culture (Figure 9A). Positive controls showed significantly increased levels of TNF-α secretion both after 24 and 48 hours (p<0.001). J774A.1 macrophages cultured on either type of multilayered DNA-coating showed TNF-α secretion levels comparable to negative controls after 24 hours of culture. However, after 48 hours of culture, significantly decreased levels of TNF-α secretion were observed on both types of multilayered DNA-coatings (p<0.001) (Figure 9B). Positive controls showed significantly increased levels of TNF-α secretion both after 24 and 48 hours (p<0.001). No differences were observed in the secretion of the cytokines IL-1β, IL-10, and TGF-β1 between the coated substrates and negative controls, however positive controls showed a significant increase in secretion for IL-1β and IL-10, but not for TGF-β1.

It has been demonstrated in the literature that rough surfaces in the micrometer regime increase the secretion of TNF-α. Additionally, a synergistic effect of surface roughness and LPS-stimulation by the increased secretion TNF-α has been observed. In our experiments, the increased surface roughness of multilayered DNA-coatings, although in the nanometer regime, evidently did not increase cytokine secretion. On the contrary, both types of multilayered DNA-coatings had a decreasing effect on TNF-α secretion. The results of the current study indicate that multilayered DNA-coatings reduce the pro-inflammatory response of macrophages as reflected by the decreased secretion of TNF-α, however, it remains to be seen whether the effects observed under the conditions of the current model system (cell density, \textit{in vitro} setting) can be fully extrapolated to \textit{in vivo} situations, in which the balance between many different cytokines and responsive cells determines the course of the biological processes.
Chapter 4

4.4.4 Surface modifications and film functionalization

The above presented studies are predominantly focused on the application of the multilayer DNA-based coatings on soft tissue implants. To investigate the potential usage on a hard tissue implant, osteblast-like bone forming cell behavior was studied on unfunctionalized coated substrates and on calcium phosphate (CaP) or BMP-2 enriched substrates.

CaP in the form of carbonated apatite is the most abundant mineral constituent in bone and pre-deposition of this material on DNA-based coated substrates is known to influence the behavior of osteoblast-like cells. Furthermore, we anticipated that the high content of phosphate groups within the molecular backbone structure of DNA, might lead to the deposition of increased amounts of CaP on the surface of coated substrates when soaked in simulated body fluids (SBF). The formation of calcium phosphate depositions on biomaterials after immersion in SBF is a well defined method to obtain a first indication about the potential bone bonding properties of biomaterials in vivo.
Figure 11. Scanning electron micrographs of Ti, Ti-[PDL/DNA], and Ti-[PAH/DNA] substrates after immersion in SBF for a period of two weeks. Images on the left were obtained by tilting the samples (approximately 60°). Higher magnifications (of non-tilted samples) are presented in the right panels.

Figure 10. Calcium deposition on Ti (controls; white bars), Ti-[PDL/DNA] (grey bars), and Ti-[PAH/DNA] (black bars) after immersion in SBF. (* p<0.05 compared to controls Ti).
Immersion in SBF₁ (standard SBF solution) did not result in deposition of CaP on any of the substrates, however immersion in SBF₂ (double Ca²⁺ and PO₄³⁻ concentration compared to SBF₁), which contains the double amount of calcium and phosphate ions, resulted in substrate-dependent deposition of poorly crystalline carbonated apatitic spheroids. Two weeks after immersion, the complete surface of Ti-[PAH/DNA]₅ was covered with a thin layer of spheroids. In contrast, the deposition of spheroids on Ti and Ti-[PDL/DNA]₅ was found to be directed away from the substrate surface, resulting in higher deposition spots (islands) rather than a complete surface coverage (Figure 10). The amount of calcium deposited on the experimental surfaces appeared to be significantly higher on both types of DNA-coatings (Figure 11). Although the film thickness of [PAH/DNA]-coatings is less than 10 nm in many parts on the surface, as was shown by XPS and AFM analysis (section 4.3), complete coverage of CaP deposition does indicate that a coating is present even on the thin sections of the film.

Cell culture experiments with osteoblast-like cells revealed that non-SBF-immersed DNA-coatings did not change osteoblast-like cell proliferation, differentiation and morphology. Pre-treatment of DNA-coatings in SBF₂ was found to affect osteoblast-like cell differentiation, as evidenced by the increased deposition of osteocalcin in the extracellular matrix. In addition, SEM showed more pronounced extracellular matrix formation, especially for Ti-[PDL/DNA]-2w substrates (Figure 12). These results are indicative of bone-bonding capacities of the DNA-coatings. Nevertheless, future animal experiments are required to provide conclusive evidence for the bioactivity of the DNA-coatings.

![Osteocalcin deposition in extracellular matrix by osteoblast-like cells cultured on Ti-[PDL/DNA]-1w, Ti-[PDL/DNA]-2w, Ti-[PAH/DNA]-1w, Ti-[PAH/DNA]-2w, and controls after 8, 16, and 24 days. Bars represent mean ± SD of one representative experiment out of 3. (* p<0.05; ** p<0.01 compared to controls [Ti]).](image-url)
To further investigate the possibility of film functionalization and to study the effect of growth factor additives on cells, multilayered DNA-coatings were functionalized with the osteoinductive factor BMP-2 using 3 different loading modalities: superficial (s), deep (d), and double-layer (dl), depending on the location of the BMP-2 (Figure 13 A). Unless the BMP-2 was applied on top of the multilayered DNA-coatings (for s and the final layer of dl), substrates were washed in ultra-pure water, after which the build up of the coatings was continued. The amount of loaded BMP-2 in and its release from the multilayered DNA-based coatings was determined using radio labeled (125I) BMP-2.

![Schematic representation of the different loading modalities of multilayered DNA-coatings with BMP-2. Note the difference in BMP-2 (dark spheres indicate drop cast BMP-2 without subsequent wash steps; white spheres indicate incorporated BMP-2 with subsequent wash steps during build-up of the coatings).](image)

**Figure 13. A.** Schematic representation of the different loading modalities of multilayered DNA-coatings with BMP-2. Note the difference in BMP-2 (dark spheres indicate drop cast BMP-2 without subsequent wash steps; white spheres indicate incorporated BMP-2 with subsequent wash steps during build-up of the coatings). **B.** Incorporation of rhBMP-2 into multilayered DNA-coatings. The amount of gamma radiation of s-loaded coatings was artificially set at 100 ng, after which d- and dl-loading were calculated accordingly. Results are presented as mean ± SD (n=3). (Loading efficiencies are 100, 15, and 32% for s-, d-, and dl-loading, respectively)

It was shown that BMP-2 was incorporated proportionally into the multilayered DNA-coatings as: $s + (4 \times d) = dl$ (Figure 13B), which illustrates that only 15% of the applied BMP-2 remained on the surface or diffused into the film after washing. The release profiles of all differently-loaded multilayered DNA-coatings showed an initial burst release, followed by a sustained release of the remaining BMP-2 for (at least) 8 weeks. As can be expected, the burst release of deep loaded BMP-2 was relatively low (35-50%) and high for superficial and double-layer loading (60-75%), which is, in light of the loss of BMP-2 upon washing, most likely caused by removal of the BMP-2 deposited on the surface. Although, a sustained release is observed for at least 8 weeks, in most cases half or more of the incorporated BMP-2 is lost within the first 24 hours. For controlled drug delivery application this needs to be improved.
Nevertheless, *in vitro* culture experiments with rat bone marrow-derived osteoblast-like cells demonstrated that the loaded factor remained biologically active, as an accelerated mineralized matrix deposition expressed as calcium amount was observed on s- and dl-loaded multilayered DNA-coatings after 12 days culture (Figure 14), without affecting cell proliferation.

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 14.** A. Mineralization (calcium deposition) by bone marrow-derived osteoblast-like cells on differently-loaded multilayered [PDL/DNA]-coatings. B. Same as A for [PAH/DNA]-coatings. (* p<0.05; *** p<0.001 compared to control).**

"Again no increase in cell proliferation could be observed"
4.5 Conclusions.

Using the LbL technique it was possible to construct DNA-based coatings using PDL or PAH as the cationic component. Analysis of the coatings on various types of substrates showed that in both cases a DNA containing coating was formed, however, the build-up of the [PDL/DNA]-coating, although based on exponential growth, was more regular in thickness than the [PAH/DNA]-coating, which was based on linear growth. XPS indicated that both types were of a mixed composition, in which the [PDL/DNA]-film surface was enriched in DNA compared to [PAH/DNA]-films after the deposition of a DNA terminating layer. The biological assays proved that both coatings are cyto- and histocompatible and displayed an increased effect on cell proliferation (RDF cells), calcium deposition (after SBF2 immersion) and cell differentiation (osteoblast-like cells on SBF-treated coatings). Furthermore, a decrease in the pro-inflammatory cytokine TNF-α was induced by the presence of the coating, providing a first indication that DNA indeed reduces the inflammatory response as was the objective of this research. In addition, the coating could be enriched with BMP-2, which was again released upon cell culture experiments thereby influencing osteoblast-like cells depending on the site of loading within the coating structure.

These results demonstrate that DNA-based LbL films are promising candidates for application as biomaterial coating, however some issues need to be addressed further. The surface of the coating is of a mixed composition and it is therefore difficult to relate the observed biological effects solely to the presence of DNA. It is therefore imperative to construct a truly layered film in order to fully exclude the influence of the cationic polymer components. A surface exclusively covered with DNA may enhance or diminish the observed effects, demonstrating which component is responsible for them. Furthermore, the loading of additives in these polymer based DNA-coatings results in a poor loading efficiency after washing and a burst release within the first 24 hours. In order to use LbL DNA-based coatings also as drug delivery systems, control has to be obtained over the release characteristics of the additives. As already mentioned in chapter 2, LbL films based on only polymer components are subject to diffusion resulting in a mixed film, as is also the case with the [PDL/DNA] and [PAH/DNA] films reported in this chapter. Control over the diffusion of the individual components of the film is key in obtaining control over the structure of the LbL coating. Hydrogen bonded self-assembled cationic surfactant aggregates will be used for this purpose in the next chapters.
4.6 Experimental

**General materials and equipment.** Polyanionic Salmon Sperm DNA (~300 bp/molecule; sodium salt) was kindly provided by Nichiro Corporation (Yokosuka-shi, Kanagawa prefecture, Japan). Potential protein impurities in the DNA were checked using the BCA protein assay (Pierce, Rockford, Illinois, USA) and measured to be below 0.20% w/w. Polycationic polyelectrolytes poly(allylamine hydrochloride) (PAH; MW ~70000) and poly-D-lysine (PDL; MW 30000 – 70000) were purchased from Sigma. All solvents were purchased from Acros Chemica or Sigma-Aldrich and of p.a. quality. Aqueous solutions were always prepared using ultra pure Milli-Q water (Barnstead EASYpure LF system; R >17.7 MΩ-cm). A 1 mg/mL aqueous solution of DNA was prepared by dissolving the supplied freeze dried DNA in ultra pure Milli-Q water. The 1 and 5 mg/mL ribbon solutions were prepared by suspending freeze dried surfactant 1 in ultra pure milli-Q water and subsequent heating to 90 °C, resulting in a clear solution. Cooling to room temperature yielded the turbid ribbon solution. Glass and quartz substrates were cleaned in Piranha solution (H2O: (30% aqueous solution) / H2SO4 – 3:7 v/v), thoroughly rinsed with ultra pure water, and dried using a pressurized air stream. Silicon substrates (1 x 1 cm; Wafernet GmbH, Eching, Germany) were coated with a 50 nm thick titanium layer, using the RF magnetron sputter technique. The titanium sputtering process (duration 5 min., pressure ~5 x 10⁻⁴ mbar) was performed with the silicon substrates attached to a rotating sample holder. Titanium-sputtered silicon substrates and titanium discs were cleaned ultrasonically using consecutively nitric acid (10% v/v), acetone, and isopropanol. Between each cleaning step, the substrates were rinsed with ultra-pure water and dried in air after the last cleaning step. UV-vis spectra were recorded on a Shimadzu MultiSpec-1501 equipped with a diode-array detector. XPS measurements were performed using a non-monochromized VG Escalab 200 spectrometer equipped with an aluminum anode (AL KR; 1486.6 eV) operating at 510 VA with a background pressure of 2 x 10⁻⁶ mbar. Spectra were acquired at 0° and 60° with respect to the surface normal. AFM images were recoded using a Nanoscope IIIa AFM set-up (Digital Instruments, Buffalo, NY, USA). All scans were made in constant mode, using a Si/Ni-tip. FTIR-spectra on quartz substrates were obtained using Perkin Elmer FTIR (Bucks, England, UK). The infrared spectrum of DNA (as sodium salt) was obtained using the KBr-pellet method (0.4 % w/w DNA in KBr).

**LbL deposition and chemical analysis**

**Coating deposition.** Multilayered DNA-coatings were generated on quartz, titanium and glass substrates. The cleaned substrates were immersed in an aqueous solution of either PDL (0.1 mg/ml) or PAH (1 mg/ml) for 30 minutes, allowing sufficient time for the adsorption of the first cationic polyelectrolyte layer onto the substrates. Subsequently, the substrates were washed in ultra-pure water (5 minutes, continuous water flow) and dried using a pressurized air stream. Thereafter, the substrates were alternately immersed in an anionic aqueous DNA solution (1 mg/ml) and in the respective cationic polyelectrolyte solution for 7 minutes each, with intermediate washing in ultra-pure water (5 minutes, continuous water flow) and drying steps using a pressurized air stream. The build-up of the multilayered DNA-coatings was continued until a total of 5 double-layers was reached. These were designated either [PDL/DNA]₅ or [PAH/DNA]₅ (the denotation of coatings is restricted to indicating the number of double-layers, i.e. ½ represents only the cationic part of the double-layer).

**UV-Vis.** The progressive build-up of the multilayered DNA-coatings onto quartz substrates was monitored using a UV-Vis spectrophotometer. Measurements were taken after each successive layer. Growth of the multilayered DNA-coatings was studied via the increase in the 260 nm maximum absorption of the nucleic base chromophores in the UV-Vis spectra. All measurements were replicated in a separate second run.
Atomic Force Microscopy (AFM). The morphologies of the non-coated titanium-sputtered silicon, partial ([PDL/DNA]_i) and [PAH/DNA]_i; [PDL/DNA]_j; and [PAH/DNA]_j; [PDL/DNA]_k and [PAH/DNA]_k) and complete ([PDL/DNA]_i and [PAH/DNA]_i) multilayered DNA-coatings were analyzed using AFM. All scans were made in the contact mode and typical scan sizes were between 0.5 x 0.5 μm² and 5 x 5 μm². To detect potential damaging effects of the probe to the multilayered DNA-coatings, scans of the samples were always made with increasing scan size. Image analysis software (WSxM free software) was used to generate micrographs and quantitatively compare surface roughness, using Root-Mean-Square (RMS) calculations, a roughness denotation based on the standard deviations of the z-value (vertical direction).

X-ray photoelectron spectroscopy (XPS). XPS-Spectra were acquired at 0⁰ and 60⁰ with respect to the surface normal, after the adsorption of 1, 3½, and 5 double-layers of [PDL/DNA] or [PAH/DNA] onto titanium-sputtered silicon substrates. As a reference, the spectrum of a dropcast (pure) DNA film was included. The (I_{P2p} / I_{N1s}) (ratio of integrated intensities) of the experimental substrates were used to calculate the fraction of DNA (x_{DNA}) in the top (5 - 10 nm) of the measured sample, according to formula 1. As the stoichiometric carbon/nitrogen ratios for DNA, PDL, and PAH are similar, no extra correction was necessary.

\[
\frac{I_{P2p}}{I_{N1s}} = x_{DNA} \left( \frac{I_{P2p}}{I_{N1s}-DNA} \right)
\]

Radio labeling. The amount of DNA immobilized into the multilayered coatings was determined with the help of radio labeled DNA. In brief, DNA was radio labeled using ³²P-postlabeling through the T4-kinase enzyme. Non-bound label was removed via 2 successive filtrations using Sephadex^®-50 columns. The efficiency of labeling was 34%. Radio labeled DNA was added to an aqueous DNA solution with a final concentration of 1 mg/ml. Concentrations of the aqueous solutions of the cationic polyelectrolytes were as described above. Multilayered coatings were fabricated as described above onto disc-shaped glass and titanium substrates. After the completion of 1, 2, 3, 4, and 5 double-layers, substrates were taken out of the fabrication process, immersed in scintillation fluid, and counted using a liquid scintillation counter. For comparison, samples containing 100 µl of the initial aqueous DNA solution (1 mg/ml) were counted. All experimental and control samples were present in 3-fold.

Biological Assays

All biological assays were performed at, and in close collaboration with, the Radboud University Nijmegen, Department of Periodontology & Biomaterials. For the experimental procedures see the articles indicated below.

4.4.1 RDF cell proliferation, viability and morphology.35,36
4.4.2 Histological assessment.35
4.4.3 Immunological response of macrophages.37
4.4.4 Calcium deposition from SBF and in vitro osteoblast-like cell behavior.38
4.4.4 BMP-2 functionalization and in vitro osteoblast-like cell behavior.39
4.1 References.

(13) Quinn, J. F.; Yeo, J. C. C.; Caruso, F. Macromolecules 2004, 37, 6537-6543.


Bis-Ureido based surfactant aggregates -

Control in shape and size through crystal growth

Abstract

Self-assembled surfactant aggregates show a large variety in shapes and sizes. Although in some cases the shape can be predicted using the postulates of Israelachvili, it is still not fully understood what exactly governs the size of the aggregates. A fine balance between attractive and repulsive forces seems to be important and understanding these forces may lead to control over the dimensions of the aggregates. In this chapter a surfactant molecule (1), which contains a bis-ureido group incorporated in its hydrocarbon chain, is introduced. Surfactant 1 forms highly ordered ribbon-like aggregates in water, due to the presence of strong hydrogen bonds in the length direction. As a result of the highly ordered molecular structure, the ribbon aggregates do not behave like a classical surfactant system, but instead show a 2-D crystal-like growth. As a consequence, their size and morphology can be controlled by changing the supersaturation of the solution similar to what is observed for most 3-D crystals. The aspect ratios of the ribbons can be varied between 2.5 to >50, by varying only the temperature or concentration.
5.1 Introduction

In chapter 2 it was shown that assembly of layer-by-layer (LbL) films using only polymer components results in mixing of the individual polymer layers. The degree of mixing is determined by the rate of diffusion of the components within the multilayer structure and related to the either linear growth for limited diffusion, or exponential growth for substantial diffusion of these films during their preparation.\textsuperscript{1,2} Diffuse exponentially growing films always yield mixed coatings, however, since the diffusion during linear growth is limited, the resulting films, in theory, should be layered. Nevertheless, confocal microscopy still indicated a layer thickness in the micrometer regime of each individual layer.\textsuperscript{2} Chapter 4 showed that also for DNA based multilayer films, in which PAH and PDL are used as the cationic polymer component, no films truly layered on a nanometer scale can be constructed. As mentioned in chapter 2, the final application of the film will determine the most appropriate degree of separation between the two LbL components. A drug delivery compartment based on slow release relies on diffusion, hence demanding a diffuse exponentially growing film. On the other hand, timed release or exposure of e.g. growth factors at the surface requires separation of compartments and thereby a high degree of control over the diffusion and structure of the coating. The DNA/polymer coatings described in chapter 4 may mainly be applied for drug delivery; however, they lack the structural separation on a nanometer scale and as a consequence the ability to anchor molecular functionalities in a predetermined layer without preliminary diffusion out of the coating. In addition, to investigate the precise influence of DNA on the cellular response, it is necessary to ensure that DNA is the top layer, which can only be achieved when the layers are separated on a nanometer scale.

Inspired by the lamellar phases found for DNA-cationic surfactant complexes (Figure 1)\textsuperscript{8-12}, which display such a nanometer-sized layer separation, it was hypothesized that LbL deposition of DNA and stable cationic bilayer surfactant aggregates, as a replacement for the cationic polymer component, would yield truly layered films on a nanometer scale. Other benefits of using surfactant aggregates instead of cationic polymers include the the improved biodegradation and the reduced ability to induce

![Figure 1. DNA-surfactant lamellar phase\textsuperscript{11}](image)
transfection, an unwanted side reaction for this application.

Frequently, the introduction of strong hydrogen bonding moieties\textsuperscript{15-15} such as amide or urea groups has been shown to be instrumental in the structuring and stabilization of surfactant assemblies. To date these efforts have resulted in the generation of a large variety of functional objects that were demonstrated e.g. to template the deposition of inorganic materials\textsuperscript{16-20} or to have a specific interaction with cell membrane receptors\textsuperscript{21,22} and other biomacromolecules.\textsuperscript{23,24} The incorporation of bis-ureido groups in molecules and polymers gives rise to strong bifurcated hydrogen bonding interactions, resulting in added stability of the formed structures.\textsuperscript{25-36} In addition, the bis-ureido unit may be utilized to functionalize the aggregates via a modular approach through molecular recognition of other bis-ureido containing molecules,\textsuperscript{37-39} opening up possibilities to anchor functionality into individual layers on a nanometer scale as well. For these reasons, the bis-ureido group was chosen as a stabilizing unit in the design of the new cationic surfactant. The surfactants used in the initial stages of this thesis were based on amino acids, in which the protonated amine moiety was used as the cationic head group in contrast to quaternary ammonium groups. Although the constructed coatings did not meet the desired structural design, initial cell culture experiments showed a favourable toxicity. Therefore, the bis-ureido based surfactant was also designed with an amine head group and not with a quaternary amine functionality. A C4-carbon spacer was chosen between the urea groups and the terminal amine head group, since degradation would result in the formation of putrescin (1,4-diaminobutane);\textsuperscript{7} a natural degradation product.

5.2 Surfactant synthesis

Surfactant 1 was synthesized starting from 1,4-diisocyanobutane, which was coupled to hexadecylamine to form synton 2 (Scheme 1). Purification via precipitation from hexane and subsequent washing with the same solvent, was followed by coupling of 2 to mono Boc-protected 1,4-diaminobutane. After precipitation from diethyl ether and washing with this solvent, the Boc-group of 3 was removed by deprotection in 90% TFA/water solution, which yielded the desired surfactant 1 in an overall yield of 43% (yields were highly dependent on the scale of the reaction). Before use 1 was freeze dried from glacial acetic acid. However, scaling-up using this strategy was troublesome. For this reason an alternative route using CDI as the coupling reagent was developed.

In this route, mono boc-protected 1,4-diaminobutane was reacted with CDI to yield the main building block 4 (Scheme 2). The latter was reacted with hexadecylamine to yield the

\textsuperscript{7} Oral administration rat LD50: 463 mg/kg, skin administration mouse LD50: 1750 mg/kg.
intermediate 5, which was easily purified via recrystallization from methanol. After removal of the Boc-group using TFA, a second reaction with 4 afforded the Boc-protected bis-urea compound. Large-scale purification of this compound was performed using Soxlet extraction with methanol. Deprotection of the residue using TFA/CHCl₃ and subsequent freeze drying from glacial acetic acid gave the final surfactant 1 in an overall yield of 34%. Although the overall yield of this route is less than the isocyanate route, this yield remained unchanged during scaling-up to gram scale, whereas in the case of the isocyanate-route the overall yield dropped dramatically.

Scheme 1. Isocyanate-route synthesis of 1.

Scheme 2. CDI-route synthesis of 1.
5.3 Aggregate formation

The TFA salt of surfactant 1 was suspended in ultra pure water (5 mg/ml). Upon heating to 90 °C a clear solution was formed, which after cooling to room temperature gave rise to the formation of aggregates as was concluded from the presence of a viscous milky suspension. Cryo-transmission electron microscopy (cryo-TEM) showed the formation of ribbon-like aggregates, which were remarkably well defined in width (widths between 50 and 90 nm, Figure 2A).

![Figure 2. A. Cryo-TEM image of a 5 mg/ml ribbon solution. B. AFM height image of a weeks old 1 mg/ml sample showing separate ribbons of various widths. C. Height profile of a single ribbon indicated with a white line in B. D. AFM phase image of ribbons deposited on a gold substrate clearly showing the grain structure of the underlying gold trough the ribbon features.](image)

AFM measurements on the aggregates drop cast from a one week old 1 mg/ml solution onto a glass substrate also revealed the presence of many ribbon-like aggregates, however, with a larger variation in width. Interestingly, these aggregates had lengths in the micrometer regime.
but showed almost no variation in the width over the entire length of a single ribbon (Figure 2B). Older matured solutions did show the presence of broader sheets.

The presence of only very few twisting points both in (dry) AFM and in cryo-TEM samples, suggested that these ribbons had a rigid structure. However, when the ribbons were deposited on a gold substrate, the underlying grain structure of the gold could be clearly distinguished through the ribbon structure in the phase image (Figure 2D). This shows that, although covered by ribbons, the AFM tip can still detect structural differences of the underlying substrate, implying a more flexible nature of the ribbon aggregates. An aggregate thickness of approximately 6 nm was estimated from the AFM height images (Figure 2C) and the few twist points observed with cryo TEM.

5.4 Molecular organization

Polarized IR spectroscopy both in the reflective (IRRAS) \(^{40,41}\) and in the transmission mode was used to determine the molecular orientation of the individual molecules within the ribbon aggregate. To this end, an IR-transparent silicon wafer was coated with a 3 nm gold layer to increase the adsorption of the ribbon aggregates onto the surface. These were flow aligned using a stream of nitrogen and AFM was used to confirm the alignment of the structures on a local 3x3 \(\mu\)m length scale (Figure 3).\(^{42}\)

IRRAS measurements (Figure 4A) showed a complete reduction of the carbonyl signals (1616 cm\(^{-1}\)) and large reduction of the NH (3321 cm\(^{-1}\)) signals, when comparing the ribbon aggregate to a drop cast film from chloroform of randomly oriented molecules of 1. The IRRAS technique enhances only vibrations that are oriented perpendicular to the substrate surface, whereas vibrations oriented parallel to the substrate, remain under the detection limit. The observed bands point to an orientation of the urea groups and thus of the hydrogen bonds parallel to substrate surface i.e. in the x-y plane of the ribbon aggregate. To determine the orientation of the hydrogen bonds within the x-y plane, polarized transmission IR spectroscopy was used on the flow aligned ribbons (Figure 4B). This experiment showed a decrease of the C=O (1616 cm\(^{-1}\)) and NH (3321 cm\(^{-1}\)) vibrations when the polarization direction was perpendicular to the length of the ribbons, which indicated that the hydrogen bonds were oriented predominantly along the length direction of the ribbons. The concomitant decrease of the CH-sym vibration (2848 cm\(^{-1}\)) both in the reflection and transmission mode suggests that in these ribbons the hydrocarbon chains are also highly ordered.

Although the observed reduction in transmission IR was not 100%, this does not mean that the alignment of the hydrogen bonds within a ribbon is not perfect. On the contrary, the
maximum angle of variation between the aligned ribbons was determined in the 3x3 μm AFM image of the flow aligned sample and amounted to ~25° (Figure 3). If one would assume perfect orientation of the molecules within a ribbon, this variation would allow a maximum reduction of 54% for the oriented IR vibrations. The observed 60% reduction nicely matches the variation found in the alignment of the sample. The observed alignment is in good agreement with the orientation of the H-bonds along the length direction of the ribbons, given the fact that the spot size of the IR beam is in the order of 25 μm.

**Figure 3.** AFM amplitude images showing local μm alignment of the ribbons, with the white arrows indicating the variation between the two extreme orientations.

**Figure 4.** A. IRRAS spectrum of a randomly oriented drop cast solution of 1 and a flow aligned ribbon sample. B. Polarized transmission IR spectrum of a drop cast flow aligned ribbon sample, with the polarization direction parallel and perpendicular to the flow direction.
X-ray diffraction experiments (Figure 5A) were conducted in order to further elucidate the molecular packing. Grazing incidence X-ray diffraction, which provides information on repeating distances perpendicular to the substrate, on a layer of horizontally deposited ribbons showed a main first order reflection at 57 Å. This d-spacing matched the estimated 6 nm thickness of the ribbon from AFM and cryo-TEM and can, through molecular modelling, only be constructed from a head-to-head distance of an interdigitated bi-layer of surfactant molecules, in which the C16 alkyl chains are fully overlapping. Powder X-ray diffraction (PXRD) on a more concentrated sample (40 mg/ml) showed two additional first order reflections at 3.9 Å and 4.5 Å.

Fitting these data to a 2-D crystal structure with the 3.9 Å d-spacing placed at an angle of 125° with respect to the 4.5 Å spacing, with minimal overlap of the Vanderwaals-radii gave two molecule-to-molecule distances, i.e. 4.76 Å and 5.4 Å (Figure 5B). The former distance corresponds well to H-bond distances commonly found for bis-urea based systems. In addition these lattice parameters represent a molecular area of 21 Å², which nicely agrees with data derived from molecular modelling and Langmuir experiments presented in chapter 7. The resulting model (Figure 6) shows a densely packed array of molecules interconnected by H-bonds along the long y-axis of the ribbon with their alkyl tails interdigitated pointing alternatingly up and down along the x-direction, forming a 2-D crystal with an elongated hexagonal pattern.

Figure 5. A. Grazing incidence XRD on flat deposited ribbons showing a major first order reflection at 5.7 nm corresponding to the thickness of the ribbon aggregates. Inset: PXRD on a 40 mg/ml sample showing 2 additional first order reflections corresponding to the d-spacing of the molecular packing. B. 2-D crystal lattice corresponding to the observed PXRD reflections under a 125° angle; white spheres are head groups pointing up and the arrows along the head groups of identical shade indicate the hydrogen-bond direction.
Variable temperature (VT) in situ AFM measurements both in water and in the dry state showed that upon heating to 70 °C the ribbons disintegrated (Figure 7). This is in line with the visual inspection of a turbid ribbon solution, which upon heating to 70 °C turns clear, also indicating the dissolution of the aggregates. The turbid mixture was again obtained upon cooling below 70 °C, indicating reformation of the ribbon aggregates, which was confirmed by AFM and TEM. DSC measurements on a concentrated gel (12.8 mg/mL) showed two endotherms: a small -0.3 kJ/mol peak (onset at 67.7 °C, max. 69.5 °C) and a larger -2.2 kJ/mol peak (onset 72.9 °C, max. 75.6 °C). These endotherms can relate to the melting of the alkyl tail segment followed by the total disintegration of the ribbon aggregates and inter-molecular hydrogen bond breakage. Although no further evidence of this assumption is available, it is however clear that the ribbon aggregates are dissolved at temperatures above 75 °C.

Figure 6. Molecular model constructed using the Materials Studio software programme showing no overlap of vanderwaals-radii. A. Side view of the unit-cell along the y-direction. B. Same as A along the x-direction. C. Top view of A along the z-direction. D. 3-D POV-Ray image of the proposed molecular packing within the ribbon structure.
VT-IR spectroscopy showed no significant changes in the CH₂ symmetric (2849 cm⁻¹) and asymmetric (2921 cm⁻¹) stretch vibrations, nor in the C=O stretch vibration (1602 cm⁻¹) between 20-70 °C, indicating that no changes in the molecular packing occurred in this trajectory. D₂O was used as a solvent in order to study the shift of the carbonyl vibration at 1602 cm⁻¹, which otherwise would be obscured by the H₂O signal. In addition, the position of the CH₂-deformation band can be used to assess the organization of the hydrocarbon chains. Gauche conformations give rise to a band at low wavenumbers (~1463 cm⁻¹), whereas a highly packed all-trans state leads to a vibration at 1471 cm⁻¹. The observed position of the deformation band (1471 cm⁻¹) indeed indicated an all-trans conformation, which upon heating to 70 °C showed no shift to lower wavenumbers. Only above 70 °C a broadening of the signals was observed which was attributed to the melting and concomitant dissolution of the ribbons.

![Figure 8](image)

**Figure 8.** In situ AFM images measured in a fluid cell showing the dissolution of a single ribbon above 70 °C as indicated by the white arrows.

### 5.5 Size control

Cryo-TEM showed that upon increasing the concentration from 0.25 to 10 mg/ml the width of the ribbons in freshly prepared solutions decreased, whereas their lengths remained unaffected (Figure 9). In addition, as the width of the ribbons decreased, the corresponding size distribution also became narrower. This is in contrast to the behavior generally observed for micellar systems, in which the size of the aggregates and their size distribution increases when the concentration is increased. Because the critical micelle concentration (CMC) is constant for a given temperature, increasing the concentration in a classical surfactant system will not result in an increase in the concentration of free surfactant molecules. Thus, above the CMC all extra added molecules are taken up in the aggregates already present or lead to the formation of new ones. This results in a broadening of the size distribution and an increase in the size of existing aggregates. Remarkably, for the present system the observed trend is completely the opposite. In fact, the observed narrowing of the ribbons formed at higher concentration shows an
analogy to crystal growth, in which a higher nucleation rate at higher surfactant concentrations results in a smaller average width and a more narrow size distribution. The dense 2-dimensional crystal-like packing of the surfactant molecules as presented in the previous paragraph and the observed concentration effect on the width of the ribbons, suggest an aggregate formation mechanism similar to that found in crystal growth.

Figure 9. A. Plot of the decreasing average ribbon width versus the increasing surfactant concentration (0.25, 0.5, 1.0, 2.5, 5.0, 10.0 mg/ml). B. Cryo-TEM images corresponding to A.
The crystalline nature of the ribbon aggregates and the presence of H-bonds that are only directed along the length axis prompted us to try and tune the aspect ratio of the ribbons through variation of the growth conditions. Therefore, a 0.25 mg/ml surfactant solution was prepared in a closed vial and heated above the formation temperature (70 °C) to 90 °C. Subsequently, the aggregates were allowed to grow by keeping the solution at 65 °C for 1 week. AFM and TEM showed the formation of faceted 6 nm thick crystal-like aggregates whose average aspect ratio (a.r.: length/width) was 2.5 (Figure 10A); a dramatic decrease compared to the ribbons grown at room temperature (a.r. >50). Irrespective of their size almost all of these aggregates showed faceted top ends with an average top angle of 125 degrees (N=80) reflecting their organization on a molecular level (Figure 10B).

![Figure 10 A](image1.png)

**Figure 10. A.** AFM image of ribbon aggregates grown at 65 °C for 1 week, showing a clear change in morphology when compared to ribbons grown at room temperature. The average aspect ratio amounts to 2.5 and the top ends clearly show facets at 125 degree angles. Inset: enlarged smaller aggregate, which has the same morphology as the larger micrometer-sized aggregates. **B.** Aggregate 2-D crystal lattice, the red line shows the 125° faceted top end for crystal-like aggregates and the black line a 90° straight top end for ribbon aggregates.

In order to further investigate the level of control that can be achieved over the aspect ratios, the aggregates were grown at different temperatures between 25 and 68 °C. Strikingly, the aggregates formed below 55 degrees were all ribbons (i.e aggregates with a.r. > 50), whereas those formed at higher temperatures displayed crystal-like morphologies (Figure 11C). The high lengths of the ribbons caused gellation of the samples, and hampered the accurate
determination of their aspect ratios. Nevertheless a decrease in ribbon length was apparent from those that were grown at higher temperatures (between 45-55 °C).

Figure 11. Box-whisker plot of the length and width distribution of aggregates at increasing supersaturation accomplished by decreasing the aggregate formation temperature or increasing the concentration of 1. Inset shows the distribution of the aspect ratios. The following statistical values are displayed: centre bar: the median; the small square: the average; the box: 50% of all data points around the median with 25% above and 25% below; between the error bars: 95% of all data points; between the whiskers: 99% of all data points; the small dash: upper or lower extreme. A. Size distribution as a function of temperature (for each T: N>100). B. Size distribution as a function of concentration (for each conc. N>100). C. AFM images corresponding to A. D. TEM images corresponding to B.
For the aggregates grown at temperatures >55 °C a clear decrease in length was observed at increasing temperatures and, interestingly, also the distribution in lengths became smaller at higher temperature. In contrast, no proportional effect was observed on the widths of the aggregates, which varied between 200 and 850 nm at all temperatures (Figure 11A). The apparent decrease in aspect ratio of the aggregates found at higher temperatures is therefore mostly due to a decrease in length. The facetted top ends were found to be present for all crystal-like aggregates, but the 125° angles were better defined for those grown at the highest temperatures. The change in morphology and the formation of facets indicate a different growth mechanism at higher temperatures compared to the one at lower temperatures by which ribbons are formed. As VT-FTIR had already indicated, no significant changes in the hydrogen bonding interactions within the ribbons occurred upon increasing the temperature. The differences in aggregate morphology found at different growth temperatures are therefore tentatively attributed to changes in supersaturation level.

To verify this hypothesis, fresh samples were prepared at room temperature with surfactant concentrations ([I] = 0.05 and 0.1 mg/ml) lower than those used for cryo-TEM (vide supra). These were investigated with conventional TEM, which indeed revealed the formation of facetted aggregates (Figure 11D). As expected, the crystals grown at 0.05 mg/ml showed a smaller average aspect ratio than those formed at 0.1 mg/ml, which again was the result of an increasing length (Figure 11B). These results show that both temperature and surfactant concentration can be used to switch between a ribbon and a 2-D faceted crystal-like morphology, but also to tune the aspect ratio of the aggregates. Moreover, the observation that the aggregate morphology can be changed in the same fashion using both temperature and concentration supports the suggestion that this effect is driven by the supersaturation rather than by the H-bonding strength, which is only controlled by temperature. Still the change from crystal-like to ribbon-like aggregates at increasing supersaturation levels implies that at higher supersaturation the growth in the length direction of the aggregates is accelerated. Although it is tempting to relate this observation to the increased influence of the H-bonds to direct the assembly of the molecules, it should be stressed that we have no experimental evidence supporting this suggestion.

The change in top end morphology from faceted to flat is not likely to be related to the formation of a different facet type with a 90 degree angle (Figure 10B). Close examination of many top ends revealed slanted, rounded, fragmented and unfinished ends (Figure 12A/B), which are indicative of roughening effects as are found frequently for 3-D crystals at higher

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† For such a crystal plane at least one of the hkl indices should be 3. High indices crystal planes are almost never observed in crystal morphologies.
supersaturation levels. Since thermal roughening is related to an increase in the entropy term as a consequence of the temperature being increased ($\Delta G = \Delta H - T\Delta S$), the formation of roughened ends in this case, i.e. with decreasing temperature, cannot be assigned to such a process. The disappearance of faceted ends as a result of increasing supersaturation, therefore must be the result of kinetic roughening.\textsuperscript{54} In addition, transport limited crystal growth can cause a second roughening effect: the formation of dendritic and dagger-shaped structures. If the growth rate of a crystal plane exceeds the rate of transport of molecules to the crystal surface, no stable facets can be developed and the plane will grow in the direction in which free molecules are still available, resulting in needle-like or dendritic outgrowths.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure12}
\caption{Roughening effects showing rounded, slanted, strait and unfinished top ends for A. images in AFM, B. TEM images. C. Single “dagger” shaped ribbons and D. many “dagger” shaped ends on a single broad ribbon characteristic of transport limited growth. Arrows indicate pointed top ends. (All aggregates have been grown at a 0.25 mg/ml surfactant concentration and at temperatures below 55 °C.)}
\end{figure}
These structural features could also be found, although not frequently, in some samples grown at temperatures below 55 °C (Figure 12C/D), highlighting again that the observed morphological features variety can be explained using crystal growth mechanisms. Although facetted top ends are observed at lower supersaturation, ageing of the ribbon morphology with roughened top ends in their mother liquor does not lead to the development of facets in time. The analysis of the crystal-like aggregates at different time points showed that the aspect ratios present at the early points in growth remained unchanged, only a smoothing of the end faces was observed.

These results indicate that the exchange of molecules between the aggregates and the solution in the exhausted mother liquor has become extremely slow. Since the crystal-like morphology formed at low supersaturation levels at early time points is nearer to the final faceted morphology, ageing of these aggregates did show the formation of better defined facets in the measured time period (~4 months). The roughened ends of the ribbon morphology formed at lower supersaturation presumably need much more time to develop facets.

5.6 Aggregate dynamics

The dynamics of the ribbon aggregates were demonstrated in a separate experiment, in which a focused electron beam of a TEM was used to create defects in the ribbons (Figure 13A). The ribbons were deposited on a special TEM grid, which was etched from a silicon wafer to yield a flat electron transparent substrate ideal for both AFM and TEM studies. When this dry sample was placed in a fluid cell in situ AFM showed the reformation of the ribbons within 10
minutes after exposure to milli-Q water at the expense of small aggregates and fragments present on the microscope grid (Figure 13B). This quick ribbon reformation at damaged sites implies a fast molecular exchange between the solution and aggregates. This therefore seemingly contradicts the slow development of facets on the ribbon morphology in aged solutions. However, it should be stressed that in the case of the experiment depicted in figure 13, fresh water is added to a dry sample, which results in a system that is not in the equilibrium state. The fast dissolution of molecules, apparent from the disappearance of the small aggregates and fragments will result in a high local molecular mobility especially at surfacewater interface. Due to the high surface free energy in the length direction (narrow on end plane along the y-direction, Figure 6A) of the ribbon and the relative low surface free energy in the width direction (long side plane along the x-direction, Figure 6B), the sudden increase in molecular mobility results in a tendency to form as many side planes as possible in order to reduce the total surface free energy of the aggregate, which can be observed as ribbon reformation and “repair” of damaged sites.

The continued growth of aggregates of 1 in solutions (0.25 and 1 mg/ml at 65 °C) that were aged for several months was also found to lead to the formation of sedimentation at the bottom of the glass vial in which the solution was stored. Analysis of the sedimentation showed the presence of extremely large sheets with dimension even into the submillimeter domain. AFM analysis on these large sheets, with high contrast in TEM, revealed an aggregate thickness of 30 nm (5 bilayers). This indicates that, when given enough time, growth in the z-direction, in which the growth rate is even slower than in the width direction, can occur (Figure 14A/B). It is remarkable that in the sedimentation large sheets are observed, that even show growth in the slowest z-direction: an increase in aggregate thickness. This contradicts the finding that, as was described in the section 5.7, the growth rate of the aggregates in the above supernatant is extremely slow as the solution ages. A possible explanation can be found in surface induced nucleation from the bottom of the glass vial in which the aggregates have been grown. Preliminary experiments with a glass plate placed at the bottom of the vial during the growth experiment showed large continuous bilayers in AFM deposited on the surface indicating that surface-induced nucleation does occur readily, which can account for the increased growth rate and the growth in even the z-direction for aggregates developing at the bottom of the vial.
5.7 Concluding remarks

Self-assembled surfactant aggregates are known to form a large variety of aggregates with different shapes and sizes, such as micelles, vesicles, rods, tubules and sheets. In several cases these aggregates can further assemble into larger hierarchical architectures. Since surfactants have a great potential as materials for various biomedical applications, it is important to obtain control over the morphology and the dimensions of the self-assembled objects. Filter extrusion, the application of ultrasound and freeze-thaw cycles are frequently utilized as techniques to change the size of micellar and vesicular aggregates. For block copolymer amphiphiles, additionally, the block lengths of the various polymer components can...
be modified, resulting in either a change in morphology or a change in aggregate size. Furthermore a number of reports in the literature describe variations in the composition of the surfactant or the mixing of different surfactant molecules in various ratio’s as a technique to achieve control over the architectures. Also variations in the solvent composition or the use of additives have been applied. Although in some cases the type or morphology of the self-assembled structure can be predicted from the molecular shape of the amphiphilic constituent using the postulates of Israelachvili, in general the factors determining the size and shape of the assembly are not fully understood.

Therefore, it is remarkable that the aspect ratio of the bis-ureido based aggregates of surfactant 1 can be controlled, without the use of the above mentioned procedures. This control comes from the fact that, unlike other surfactants, molecules of 1 assemble into a 2-D crystal-like arrangement driven by a combination of strong hydrogen bonding between the bis-ureido units and the hydrophobic interactions between the hydrocarbon chains. These aggregates therefore, behave similarly as 3-dimensional crystals and show facetted growth at low supersaturation and roughened growth at high supersaturation. The growth rate in the length direction is most affected by the change in supersaturation, however, at this point an effect of the hydrogen bonds, which are arranged in this direction cannot be excluded nor confirmed. The mechanism of self-assembly is most likely based on a molecular growth process rather than on a process involving fusion of smaller aggregates, based on the observation that no large sheets are formed upon film dehydration and on the quick reformation of damaged sites. Due to the similarity with crystal growth it was possible to apply concepts from the crystal growth field to the formation of these surfactant aggregates and to tune their shape and size through variation of the growth conditions, i.e. the degree of supersaturation. The ability to control the shape and size of the bilayer aggregates could be used to grow large sheets, which may also be further promoted by surface induced nucleation. These sheets are the ideal candidates for the production of a truly layered lamellar DNA-surfactant LbL film as was mentioned in the introduction section of this chapter.

In the next chapter we will discuss the functionalization of both the ribbons and the crystal-like aggregates, with the help of the molecular recognition properties of the bis-urea group. While the incorporation of functionality in surfactant aggregates has been previously demonstrated also for other surfactant systems and the control over aspect ratios knows many examples in crystal engineering, the present system combines these two aspects. In particular the fact that all observed aggregates from 1 display a bilayer structure, allows, in principle, the production of 2-D objects of which not only the surface can be modified with a variety of functionalities, but also the aspect ratios can be controlled, thereby making them attractive
candidates for a wide range of applications ranging from nanotechnology to biomedical engineering.

5.8 Experimental

General materials and equipment. All reagents were obtained from Sigma-Aldrich and used without further purification. All solvents were purchased from Acros Chemica or Sigma-Aldrich and were of analytical quality (p.a.). The water used during synthesis was demineralised prior to use. Ultra pure water (Barnstead EASYpure LF system; R >17.7 MΩ·cm) was used for preparing the aggregates solutions. Deuterated solvents were purchased from Cambridge Isotope Laboratories. 1H-NMR spectra were recorded on a Varian Mercury 400 MHz instrument. Elemental analysis was performed on a Perkin Elmer 2400 apparatus. Reflection absorption IR measurements were performed on a Bio-Rad FTS 6000 spectrophotometer equipped with a Harrick Seagull™ reflection absorption unit operating at an 80° angle of incidence. For polarized transmission IR measurements the FTS 6000 was coupled to a Bio-Rad Uma 500 IR microscope with a MCT (Mercury Chromium Tellurium) detector. Both reflection and transmission experiments were recorded with a 4 cm⁻¹ resolution. The VT-IR measurements were recorded using a Bio-Rad Excalibur IR spectrophotometer equipped with a Specac Golden Gate ATR Unit fitted with a diamond window and operating with a 2 cm⁻¹ resolution. The VT-IR experiments were carried out with deuterated water as solvent to avoid overlap of the carbonyl vibration with the H₂O signal. Transmission microscopy measurements were performed on a FEI Tecnai 20, type Sphera TEM operating with a 200 kV LaB₆ filament. Images were recorded with a bottom mounted 1k x 1k Gatan CCD camera. A Gatan cryo-holder operating at ~170 °C was used for the cryo-TEM measurements. The sample vitrification procedure was carried out using an automated vitrification robot (FEI Vitrobot™ Mark III). TEM grids, both 200 mesh carbon coated copper grids and R2/2 Quantifoil Jena grids were purchased from Aurion. The Quantifoil grids were surface plasma treated using a Cressington 208 carbon coater operating at 5 mA for 40 seconds prior to the vitrification procedure. Light microscopy images were recorded on a Sondag light microscope equipped with a Polaroid camera. AFM images were recorded on a Veeco Multimode instrument equipped with a Nanoscope IV control unit using Nanosensors tips with resonance frequencies of 300 kHz operating in the tapping mode regime. For the in-situ AFM measurements the scanner was fitted with a heating stage and/or fluid cell. Powder X-ray diffraction measurements were carried out on a Rigaku Powder diffractometer (Cu-tube: CuKα radiation; λ=1.54056Å, 40 kV, 30 mA). Gracing incidence X-ray diffraction was measured on a Bruker D8 Advance instrument (CuKα radiation) equipped with a Vantec detector.

4[1-Hexadecylureido]butyl]ureido-butylammonium trifluoroacetate (1) (Isocyanate route). 1,4-Diisocyanatobutane (5.4 mL, 5.97 g, 0.043 mol, ρ: 1.106, Mw: 140.14) was mixed with 1 mL of dry toluene in a dry roundbottom flask that was fitted with a addition funnel containing 514 mg (0.002 mol, Mw: 241.45) of 1-hexadecylamine dissolved in 10 mL of dry toluene. The whole system was kept under an argon atmosphere. While stirring vigorously, the hexadecylamine was added dropwise over the course of 25 min. and left to react for an additional hour. The reaction mixture was precipitated in cold dry hexane and after filtration, the white precipitate (2) was washed 5 times with 20 mL cold hexane. Subsequently, 2 was redissolved in 20 mL of dry chloroform and passed through a glass filter to remove the last insoluble impurities. Next, 550 mg (0.0014 mol, Mw: 381.60) of 2 was suspended in dry chloroform and added dropwise to 303 µL (0.30 g, 0.0016 mol, ρ: 0.984, Mw: 188.27) of mono(N-Boc)-1,4-butandiamine dissolved in 5 mL of chloroform, while stirring vigorously and under argon atmosphere. The reaction was monitored using IR spectroscopy and stopped when the characteristic isocyanate vibration (~2250 cm⁻¹) disappeared.
After precipitation in diethyl ether and filtration, the resulting white powder (3 Mw: 568.7) was washed an additional 5 times with 20 mL of diethyl ether to remove all impurities. The Boc-group was removed by dissolving 3 in a 90% TFA/water mixture and heated for 1 h. at 60 degrees. The final product 1 (0.54 g, Mw: 583.77) was obtained by evaporation of the TFA/water mixture and subsequent freeze-drying from glacial acetic acid yielding a white fluffy powder in an overall yield of 43%. $^1$H NMR (CDCl$_3$/CD$_3$OD): δ 0.9 (CH$_3$CH$_2$, 3H, t); 1.2-1.9 ((CH)$_3$CH$_2$, 28H, NHCH$_2$(CH$_2$)CH$_2$NH, 8H m); 2.9 (CH$_2$NH$_2$, 2H, m); 3.1 (CH$_2$NH, 8H, m); NH not visible due to H-D exchange. IR (ATR): ν = 3320 (NH) 2920, 2847 (a-sym, sym-CH stretch), 1660 (C=O TFA), 1613 (C=O Urea), 1569 (Amid II), 1478, 1465 (split CH$_2$: deform.). MALDI-TOF-MS: m/e 470.44 (M$^+$); 492.25 (M$^+$ - H + Na). Anal.calcd. (%) for CsH$_{36}$F$_5$N$_2$O$_6$: C 57.6, H 9.7, N 12.0; Found (%): C 56.3, H 9.5, N 11.8.

4[4(Hexadecylureido)butyl]ureido-butylammonium trifluoroacetate (1) (Carbodiimidazole route). Mono(N-boc)-1,4-butanediamine (34.8 g, 0.185 mol, Mw: 188.27) was dissolved in 120 mL of chloroform, after which 41.71 g (0.257 mol, Mw: 162.15) of carbonyldimidazole was slowly added. The mixture was stirred for 3 h. at 0 °C under a nitrogen atmosphere. The resulting product 4 was dissolved in 750 mL chloroform and washed with 400 mL water, 500 mL brine and dried over magnesiumsulfate. After filtration, the solvent was evaporated and 25.82 g (0.091 mol, Mw: 282.34) of 4 was dissolved together with 20.34 g (0.084 mol, Mw: 241.45) of 1-hexadecylamine in 300 mL of chloroform and refluxed for 3 h. After solvent evaporation, the product 5 was recrystallized using 450 mL methanol. Compound 5 was dried in a vacuum oven at 60 °C for 2 days to remove all traces of methanol. The Boc-protecting group was removed by slowly dissolving 5 in a mixture of 100 mL TFA and 200 mL chloroform and refluxed overnight, after which the solvent was removed and the remaining product was freeze-dried form glacial acetic acid. The resulting white powder (27.54 g, 0.059 mol, Mw: 469.63) was dissolved in 250 mL of chloroform together with 16.56 g (0.057 mol, Mw: 282.34) of compound 4 and 1 equivalent of TEA to remove the TFA counter ion, and left to reflux for two days while stirring vigorously. Subsequently, the solvent was evaporated and the product was purified via Sphelx extraction with hot methanol over the course of 1 week. After cooling the product recrystallized from the extracted methanol. The Boc-group of the resulting 17.2 g (0.03 mol, Mw 569.86) pure product deprotected by dissolving the latter in a mixture of 136 mL TFA and 100 mL chloroform, which was subsequently refluxed overnight while stirring. After evaporation of the solvent the product was freeze-dried from glacial acetic acid to yield the desired pure product 1 as a white fluffy powder (16.97 g, 0.029 mol, Mw 583.77) in an overall yield of 34.4%. $^1$H NMR (CDCl$_3$/CD$_3$OD): δ 0.9 (CH$_3$CH$_2$, 3H, t); 1.2-1.9 ((CH)$_3$CH$_2$, 28H, NHCH$_2$(CH$_2$)CH$_2$NH, 8H m); 2.9 (CH$_2$NH$_2$, 2H, m); 3.1 (CH$_2$NH, 8H, m); NH not visible due to H-D exchange. IR (ATR): ν = 3320 (NH) 2920, 2847 (a-sym, sym-CH stretch), 1660 (C=O TFA), 1613 (C=O Urea), 1569 (Amid II), 1478, 1465 (split CH$_2$: deform.). MALDI-TOF-MS: m/e 470.44 (M$^+$); 492.25 (M$^+$ - H + Na). Anal.calcd. (%) for CsH$_{36}$F$_5$N$_2$O$_6$: C 57.6, H 9.7, N 12.0; Found (%): C 57.4, H 9.6, N 11.6.

Aggregate sample preparation

In general. Surfactant 1 was suspended in at the desired concentration in ultra pure water and heated up to the boiling point of the solvent resulting in a clear solution. The solution was then cooled to the desired formation temperature either in a controlled fashion or by cooling on the bench top, resulting in a turbid suspension or gel depending on the concentration of surfactant.

Crystalization experiments. For the controlled crystallization experiments, the boiling 0.25 mg/ml surfactant solution was prepared in a closed vial and placed in a dessicator, which was situated in a stove operating at 90 °C and contained water to prevent evaporation of the surfactant solution. After 45 min. equilibration, the temperature was decreased gradually in a controlled way during a 1000 min. countdown to the desired temperature of
formation. When the formation temperature was reached, the sample was left for a growth period of 1 week, after which samples were taken for AFM and TEM measurements. To obtain the 3-D crystals of surfactant 1 aggregates, the growth period was extended for a longer time period of 4 months. This method was also used for shorter growth periods down to 1 day. For growth periods within the hour range, an oil bath was used to control the temperature of formation. In those cases a boiling surfactant solution was prepared in a closed vial and placed directly in an oil bath at the desired formation temperature. Samples for AFM and TEM were prepared after 1 or 2 h.

**Transmission electron microscopy**

**Conventional.** Small aliquots (3 μL) of the sampled aqueous ribbon suspension were applied to a 200 mesh carbon coated copper grid and subsequently, excess liquid was quickly manually blotted away with filter paper. The samples were analyzed on the Sphera microscope at 200 kV and room temperature.

**Cryo.** The cryo-TEM images of the ribbons were obtained by applying small aliquots (3 μL) of the aqueous ribbon suspension at various concentrations to Quantifoil grids (R2/2 Quantifoil Jena) within the environmental chamber (relative humidity 100%) of the Vitrobot™ instrument at room 22 °C. Excess liquid was blotted away with filter paper using an automatic blotting device within the environmental chamber of the Vitrobot™. The grid was subsequently shot through a shutter into melting ethane placed just outside the environmental chamber. The vitrified specimens were stored under liquid nitrogen and observed at -170°C (Gatan cryo-holder) in the Sphera microscope. Micrographs were taken at 200 kV using low dose conditions. For the fusion experiments Quantifoil grids, which were coated with a thin carbon layer covering the holes, were also used next to the uncovered Quantifoil grids. In order to induce fusion of the ribbons, small aliquots (3 mL) of a 5 mg/ml ribbon suspension were applied to a Quantifoil grid, with and without added carbon film, within the environmental chamber of the Vitrobot™. Excess liquid was blotted away with filter paper as described above and the remaining film was dehydrated in a controlled fashion by maintaining the relative humidity at 80% for 1 h, after which the samples were shot into liquid ethane. The samples were then treated and imaged like any other cryo-TEM sample.

**Atomic force microscopy**

**Conventional.** The samples for the crystallization experiments at different formation temperatures were prepared by drop casting of the aggregate suspension directly from the temperature controlled vial onto a clean glass slide, immediately followed by rapid removal of excess fluid using a stream of nitrogen. Also other ribbon suspensions, which were analyzed by AFM were prepared using this drop casting method on any available substrate, unless indicated otherwise.

**Fluid cell and temperature control unit.** A 1 mg/ml ribbon suspension was deposited on a glass slide as described above and placed in the fluid cell fitted with the temperature control unit. The temperature was slowly raised from room temperature to 80 °C and an AFM image was recorded every 10 °C in the dry state. A new similar sample was placed in the fluid cell and imaged before and after addition of demineralized water. A single ribbon was selected, subsequently the temperature was raised again slowly from room temperature to 80 °C and an image was recorded every 10 °C. For the self-healing experiment a special atomically flat TEM grid was etched from a silicon wafer and a 0.5 mg/ml ribbon suspension was deposited on top. Subsequently, radiation damage was induced using the focused electron beam of the TEM instrument, the amount of which could be monitored by the appearance of white blisters in the TEM images. The sample was removed from the TEM and placed within the fluid cell of the AFM. A single dry state image was recorded before addition of demineralized water, after which several images were recorded in the continuous scanning mode in the same area as the recorded dry state image.
Infrared Spectroscopy

**Polarized.** An aligned ribbon sample was necessary for the polarized reflection and transmission IR experiments. To this end, an IR-transparent silicon waver, which was coated with a 3 nm thin gold layer to increase adsorption to the surface, was placed at a 45 degree angle. A droplet of a 5 mg/ml ribbon solution was moved down the waver using gravity and a stream of nitrogen, after which the waver was turned 180° and the procedure was repeated until the droplet was fully deposited. Using the IR microscope an area was selected, measured in transmission mode, marked and subsequently imaged using AFM to confirm local alignment. For reflection absorption measurements the waver was placed on a Harrik Seagull™ Reflection absorption unit and measured using polarized IR light at an 80° angle of incidence. A similar procedure was followed using a sample drop cast from a 1 mg/ml CHCl₃/MeOH molecularly dissolved surfactant solution.

**Variable temperature attenuated total reflection (VT-ATR).** A 40 mg/ml gel was prepared as described in the general sample preparation, using D₂O as a solvent. An O-ring was placed around the diamond window on top of the ATR heating stage, filled with the aggregate gel and covered with a glass slide. The spectra were recorded after an equilibration period of 5 min. at the desired temperature starting from room temperature up to 90 °C with steps of 5° below 45 °C and above 80 °C and steps of 2 °C in between this temperature range.

**X-Ray Diffraction.** The aligned ribbon sample, which was used for the polarized transmission IR experiments, was also used for the grazing incidence XRD experiment. Powder X-ray diffraction was performed on a 40 mg/ml ribbon gel, which was placed in the X-ray beam; however measurement times could only be extended to 1 h. due to heating of the sample within the beam, eventually reaching 70 °C, resulting in dissolution of the aggregates and loss of signal. Due to the short time window, no clear sharp reflections were obtained. The 40 mg/ml gel, therefore, was dehydrated completely and ground into a fine powder, which was more stable under the influence of the X-ray beam. The measured reflections using this method were significantly sharper, however still showed a maximum which corresponded to the hydrated, gelled sample.
5.9 References


(43) Similar results were found for the β’ polymorph of triacylglycerol crystals, see ref. 44, 45.
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6

Modular functionalization of Bis-Ureido based surfactant aggregates

Abstract

In this chapter we demonstrate that the bis-ureido based surfactant aggregates presented in the previous chapter can be functionalized via a modular approach through molecular recognition of other bis-urea containing molecules. The selectivity of the molecular recognition was demonstrated via incorporation of “disperse orange”, which had been coupled to a matching and as well as to a non-matching bis-ureido functionality. The functionalization was demonstrated for both the ribbon and for the 2-D crystal-like morphologies of the aggregate by incorporation of biotin, which was modified with a matching bis-ureido group. The incorporation of biotin was visualized using gold-labelled streptavidin, thereby showing that biotin retained its biological functionality when incorporated into the aggregate structure and remained accessible at the surface.
6.1 Introduction

Funtionalization of phospholipid vesicles (liposomes), with molecules of biological origin is a hot topic due to the possible application in target specific drug-delivery and imaging. In most of these cases phospholipid molecules are modified with the targeting structure itself or with a covalent coupling unit, to which the functional targeting molecule can be coupled. Amongst others, conjugation techniques like “click chemistry”, maleimide coupling and native chemical ligation have been employed to covalently functionalize preformed phospholipid vesicles with sugar fragments, B cell and T helper cell peptide epitopes as well as fluorescent proteins.

Although molecular recognition via hydrogen bonding in an aqueous environment is a frequently occurring phenomenon in biological systems, only few examples of this principle exist in synthetic systems. Most reports in the literature use the molecular recognition behaviour of known biological systems involving base pair recognition or the biotin-streptavidin interaction. One of the synthetic examples is the molecular recognition between ureido-pyrimidinone (Upy) units, which dimerize via strong quadruple hydrogen bonds. These units have been used in the construction of supramolecular polymers, which could be functionalized, via molecular recognition, with various short peptide sequences also equipped with a Upy unit. Other well known examples of hydrogen bond driven molecular recognition involve triaminotriazine derivatives and barbituric acid (derivatives), in which the latter is bound in a wedge conformation via triple hydrogen bonds to two adjacent triazine units. When studied in an aqueous environment, many of these molecular recognition studies are performed on Langmuir-monolayers situated at the air water interface, however only few examples exist in which molecular recognition is studied in bulk water via functionalization of surfactant aggregates. These systems display the self-recognition properties on the aggregate surface via modification of the recognition group with a hydrophobic tail or phospholipids fragment and therefore use hydrophobic interactions as an anchor rather than the self-recognition properties of the hydrogen bonds to functionalize the aggregate. Anchoring functionality through only molecular recognition within an aggregate structure in aqueous media has not yet been reported.

The functionalization of materials using molecular recognition between synthetic bis-urea units has been demonstrated for an organogel, and for an

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*C Coupling of an azide functionalized group to a terminal alkyne fragment
† Coupling of a thiol or terminal amine containing ligand with a maleimide functionalized unit
‡ Coupling of a C-terminal thioester to an N-terminal cysteine
aqueous amphiphilic block copolymer system. In this concept a guest molecule is equipped with a bis-urea unit with a certain spacer length between the urea groups and is incorporated into a host system carrying an identical bis-urea unit. Molecular recognition was demonstrated through variation of the spacer length in the guest bis-urea unit or via replacement of urea units in the host system by ester functionalities.

![Figure 1. Utilized bis-ureido based molecules: bis-ureido butylene based surfactant 1, bis-ureido butylene modified “disperse orange” 2, bis-ureido pentylene modified “disperse orange” 3 and bis-ureido butylene modified biotin 4.](image)

In the previous chapter the formation of well-defined self-assembled bis-ureido based bi-layer aggregates in water was discussed, with the aim of producing “truly” LbL films in conjunction with DNA, in which the individual layers are separated on a nanometer scale. The present chapter demonstrates that, apart from acting as a stabilizing unit, the bis-urea unit can be used as an anchor for functional molecules, which have been equipped with a matching bis-ureido unit. This combines the concepts of surfactant aggregate functionalization through anchoring units and molecular recognition in an aqueous environment. It is shown that the ribbon aggregates of the bis-ureido butylene based surfactant (1) presented in chapter 5 can be functionalized with matching bis-ureido butylene modified molecules. The molecular recognition concept is demonstrated via incorporation of the dye “disperse orange”, which has been modified with a matching (2) and non-matching (3) group within the ribbon morphology. It is also demonstrated that both the ribbon and 2-D crystal like aggregate morphologies of 1 can be functionalized with molecules of biological origin (biotin) (4).

Ultimately, this would allow for the enrichment of the aggregate layer of the LbL DNA-based biomaterial coating with bioactive signals such as growth factors, which due to the anchoring would only be exposed when the coating is degraded down to the functionalized layer. These functional molecules will be designed with a spacer between the functionality and
the bis-ureido anchor in order to penetrate through the surface layer of DNA, thereby exposing both the functionality as well as the DNA at the surface of the coating (Figure 2).

**Figure 2.** Schematic image of a peptide functionalized ribbon bilayer, in which the peptide fragments are anchored to the surfactant aggregate structure and are protruding into the adjacent DNA layer in an LbL biomaterial coating.

### 6.2 Molecular recognition

The molecular recognition capabilities of the bis-ureido butylene surfactant aggregates were investigated by studying the difference in UV-vis absorption spectra of the azobenzene dye “disperse orange”, which had been functionalized with a matching bis-ureide butylene (2) or a non-matching bis-ureido pentylene group (3), anchored within the aggregate structure. In order to study the effect of molecular recognition with respect to a surfactant without any bis-urea units, cetyltrimethylammonium bromide (CTAB) was used.

### 6.2.1 Dye incorporation

Both bis-urea functionalized dye molecules 2 and 3 are insoluble in water (Figure 3A). Dissolution of 2 and 3 in chloroform/methanol mixtures results in a clear orange solution. In a
two phase system composed of water and chloroform (Figure 3B), both dye molecules remain in the organic solvent and precipitate out in the aqueous phase (Figure 3C,D) after evaporation of the organic phase. For the incorporation of 2 and 3 within the ribbon structure, a 5 mg/ml solution of bis-urea surfactant 1 was prepared in chloroform/methanol (9:1 v/v), to which 6 mol% of 2 or 3 was added, resulting in a clear orange stock solution. For UV-vis measurements, these solutions were diluted with chloroform until an optical density (OD) of 1.0 was reached (46 x for 2 and 11x for 3). Addition of water to both stock solutions resulted again in the formation of a two phase system; however in this case evaporation of the organic solvent did not cause precipitation of the 2 and 3 in the aqueous phase. Instead, a yellow suspension was formed (Figure 3E) and TEM analysis confirmed the formation of ribbon aggregates (Figure 4, inset). Water was added to the remaining yellow suspension to reach a final surfactant concentration equal to the initial concentration of the stock solution in chloroform (5 mg/ml).

UV-vis spectra of the resulting suspensions were taken using the same dilution ratio as used for the stock chloroform solutions of surfactant and dye, which had been adjusted to OD = 1.0.

For the incorporation of dye in CTAB micelles, the same procedure was followed as for surfactant 1, in which 6 mol% of 2 was added to a 5 mg/ml CTAB solution in chloroform/methanol (9:1 v/v). In contrast to the ribbon suspension, addition of water and subsequent evaporation of the organic solvent resulted in the formation of a clear orange aqueous solution (Figure 3F).

Figure 3. Bis-urea modified dye 2 in various solvent mixtures: A. water, B. chloroform/water, C. chloroform/water mixture at the boiling point of chloroform, D. water after evaporation of the organic phase, E. same as D in combination with surfactant 1, F. same as E in combination with CTAB.

UV-vis spectra recorded for both 2 and 3 in chloroform/methanol (9:1) solution showed a single absorption maximum $\lambda_{\text{max}}$ at 405 nm (Figure 4). Also 2, when dissolved in the aqueous CTAB solution, showed a single band at the same wavelength. The yellow ribbon dispersion containing matching dye 2 revealed a single $\lambda_{\text{max}}$ at 354 nm, which is significantly blue shifted when compared to the aqueous CTAB solution and the modified dyes dissolved in organic solvent. Remarkably, the coexistence of two absorption bands at 340 and 405 nm was observed for the ribbon dispersion containing the non-matching dye 3. The position of the 405 nm
absorption is equal to the $\lambda_{\text{max}}$ found for organic solvents and the aqueous CTAB solution; however, the position of the second 340 nm absorption is even more blue shifted when compared to the single $\lambda_{\text{max}}$ absorption of the matching dye. In addition, both ribbon dispersions containing either dye 2 or 3 showed an increase in scattering when compared to the stock solutions in organic solvent or the aqueous CTAB solution.

![UV-vis spectra](image)

**Figure 4.** UV-vis spectra of 2 (I) dissolved in chloroform, (II) dissolved in an aqueous CTAB solution, (III) incorporated in a ribbon suspension and (IV) of 3 incorporated in a ribbon suspension. Inset: TEM micrograph of III.

In general three possible explanations are given for a blue shift in azobenzene systems: (I) polarity effects of the solvent, (II) cis-trans isomerisation, and (III) H-aggregation. As the origin of the blue shift is related to the chemical environment, further experiments were conducted to get more insight into the intermolecular interactions between dyes and surfactants, which will be described in the following section.

### 6.2.2 Polarity study

UV-vis experiments were performed using both dye molecules 2 and 3, in which the solvent polarity was gradually increased by going from 100% chloroform to 100% methanol and subsequently to water containing 10% methanol (v/v). Increasing polarity by going from chloroform to methanol caused a small blue shift for the $\lambda_{\text{max}}$ of 15 nm from 405 to 390 nm (Figure 5A). A subsequent decrease of the absorption band was observed upon increasing the water content from 0% (100% methanol) to 30 vol%. However, at 40 vol% a second absorption
peak appeared at 340 nm whereas the absorption intensity at 390 nm became largely reduced. Increasing the water content up to 90 vol% resulted in an increase of the 340 nm and a complete reduction of the 390 nm absorption, accompanied by an increase in scattering (Figure 5B). The small gradual shift of 15 nm to 390 nm is likely to be caused by an increase in polarity. However, considering its sudden character and the large change over a relative small range in polarity, the blue shift of 50 nm from 70% to 60% methanol/water is more likely caused by aggregation. This idea is further strengthened by the observed increase of scattering and concomitant decrease in absorption.

![Figure 5](image)

**Figure 5.** UV-vis spectra of 2 dissolved in increasing polar solvent. A. From chloroform to methanol; the arrows mark the small blue shift from 405 to 390 nm B. From methanol to 10 vol% methanol/water; the arrows mark the increasing blue shifted absorption at 340 nm and the decreasing 390 nm absorption. The spectra are corrected for scattering.

### 6.2.3 Isomerization and aggregation

Azobenzene dyes are well-known photosensitive chromophores because of their cis-trans isomerization by irradiation with UV or visible light, which is accompanied by a shift in absorption.\(^{30,36-40}\) The trans-isomer of the utilized azobenzene dye “disperse orange” is thermodynamically the most stable conformer and shows a \(\lambda_{\text{max}}\) at a larger wavelength compared to the cis-isomer. The half-life of the cis-isomer, at room temperature, is in the order of 1 second.\(^{36}\) Since the blue shift in the measured samples was a permanent effect and the samples were heated above 80 °C during preparation, it cannot be related to the presence of the cis-isomer.
Figure 6. Cis-trans isomerization of an azobenzene system

Figure 7. Splitting of the excited state due to interaction of the transition dipole moments of the chromophores. This results in a forbidden lower energy level for H-aggregation (higher energy level for J-aggregation), due to cancelling of the transition moments. The higher energy level (lower for J-type) is allowed resulting in a blue shift for H-type aggregates (red shift for J-type).

H-aggregation is the aggregation of parallel-orientated chromophores in which the aromatic system is stacked in a face-to-face orientation driven by highly favourable π-π interactions. H-aggregation is characterized by a blue shift originating from a splitting of the excited state of the chromophores into a higher and a lower energy level, as a result from an interaction between the transition dipole moments. The lower energy level corresponds to the interaction of antiparallel transition dipoles and is, due to cancelling of the transition dipole moments, forbidden (Figure 7). The excitation to the higher energy level is allowed, resulting in an excited state which is higher in energy than that of the monomer itself and therefore a shorter wavelength (ΔE = h/λ) band that corresponds to a blue shift of several tens of nanometers, depending on the distance between the chromophores. The opposite effect, a red shift, can also occur and is characteristic for J-type aggregation, in which the aromatic system is positioned in a head-to-tail fashion. Since the observed blue shift for both matching and non-matching dyes within the aqueous ribbon dispersion is in the order 50 nanometers, accompanied by an increase in scattering and a decrease in absorption, it is suggested that H-type aggregation is likely to be involved.
6.2.4 Discussion

Although the polarity of the solvent does affect the position of the absorption bands of both dyes resulting in shifts to shorter wavelengths, this effect is only marginal and cannot account for the observed large blue shifts of more than 50 nm that is observed when 2 and 3 are incorporated into the ribbon structure. As based on the short half-life of the cis-isomer, cis-trans isomerization can also be excluded. This leaves only H-aggregation as a possible explanation for the observed magnitude of the shift. After addition of water to the stock solution of dye and surfactant, evaporation of organic solvents forces the dye molecules to interact with the surfactant molecules due to insolubility of the dye in water. In the highly ordered ribbon structure, matching dye molecules (2) all become “locked in” in a similar fashion resulting in uniform H-aggregates accompanied by a single blue shifted absorption (Figure 8A). In contrast, the non-matching dye 3 does not fit the ribbon lattice and is therefore less tightly bound resulting in a higher mobility of the molecules. The band at 340 nm therefore is attributed to phase separation of 3 at the ribbon surface due to inefficient anchoring within the ribbon lattice, causing a more effective H-aggregation and a larger shift (cf. 354 nm for 2)(Figure 8B). CTAB forms micelles, which are much smaller in diameter than the ribbons of 1, resulting in
less scattering and a clear solution. The dye molecules, due to their hydrophobic nature are taken up in the apolar inner core of the micelle. No aggregation occurs in the micelle resulting in no change of the $\lambda_{\text{max}}$ compared to apolar solvents. Because $\text{3}$ showed a residual absorption maximum at 405 nm when incorporated in the ribbon suspension, it is proposed that a part of the dye molecules, also due to ineffective anchoring, is taken up into the hydrophobic inner part of the ribbon (Figure 8B). These combined observations indicate that matching dye $\text{2}$ is more effectively bound into the ribbon structure when compared to the non-matching dye $\text{3}$. It is however apparent, that both water insoluble dyes have been incorporated into the ribbon structure given the similarity of the resulting spectra when compared to the spectra of the neat dye molecules in water/methanol mixtures containing $<40$ vol% water.

### 6.3 Biotin functionalization

**Figure 9.** A. Schematic representation of bis-urea modified biotin ($\text{4}$) incorporated into the aggregate structure and incubated with gold-labeled streptavidin. B. TEM image of biotin functionalized ribbons with 25 nm gold-labeled streptavidin selectively bound to the pendant biotin groups. Inset shows a larger magnification of the highlighted area.
In order to demonstrate the possibility of functionalizing the ribbon aggregates with molecules of biological origin, biotin was coupled to a matching ureido-butylene moiety via a poly(ethylene glycol) PEG spacer. The presence of biotin can be confirmed via a conventional immuno labeling assay using gold-labeled streptavidin (Figure 9A). Following the procedure described in section 6.2.1, 10 mole% of 4 was incorporated into aggregates of 1 at a 1 mg/ml surfactant concentration. TEM analysis of the resulting aqueous dispersion showed also in this case the formation of ribbons. Incubation of the biotin-containing ribbons with gold-labeled streptavidin led to the selective decoration of the ribbons with these biomacromolecules (Figure 9B), with gold particles only sporadically present in the background. Non-specific binding was checked by incubation of ribbons containing only 1 with the gold-labeled streptavidin, which yielded only a very low amount of ribbon bound gold particles indistinguishable of the gold particles present in the background.

Energy dispersive X-ray analysis (EDXA) on the incubated ribbons was performed in the STEM mode combined with the EDX detector to confirm the presence of gold. When the scanning area was positioned away from the gold particles, no gold reflections could be observed (Figure 10A). However, when the scanning area was positioned on top of the gold particles clearly all reflections characteristic for gold could be observed, which were intensified when the scanning area was focused on a single particle (Figure 10B).

![Figure 10](image)

**Figure 10.** EDXA of 25 nm streptavidin labelled gold particles attached to biotin functionalized ribbons. **A.** Bright field image and corresponding EDXA, with the square indicating the scanning area where gold particles are absent. **B.** Same as A, with scanning area reduced to a spot focused on a single gold particle.

From close examination of gold particles bound to biotin functionalized ribbons it is not clear if the particles show a more preferred binding to the edge of the ribbon aggregates or to the center. To exclude the preferential binding of 4 at the ribbon edges as well as to
demonstrate the possibility of functionalizing both aggregate morphologies of 1 (i.e. both the ribbon and the crystal-like aggregates), a 0.1 mg/ml surfactant concentration was used to prepare the biotin-functionalized aggregates. It was shown in chapter 5 that the aggregates formed at these low concentrations are of the 2-D crystal-like morphology. These aggregates are broader than their ribbon counterparts, which would lead to a more clear accumulation of gold particles at the aggregate edges when binding to the center of the aggregates would not be preferred. On the contrary, a highly selective binding to the ribbon surface was observed without any indication of preferred binding to the edges when the biotin incorporated 2-D crystal-like aggregates were incubated with gold-labeled streptavidin (Figure 11A).

![Figure 11. A. TEM image of biotin functionalized 2-D crystal-like aggregates with, homogeneously distributed, 15 nm gold-labeled streptavidin selectively bound to the pendant biotin groups. Inset shows a larger magnification of the highlighted area. B. TEM image of the control sample, in which 4 was not incorporated in the aggregate structure, showing sporadic randomly distributed gold conjugates.](image)

Furthermore, the distribution of gold particles on the surface of the 2-D crystals was homogenous and no signs of phase separation were observed. Incubation of unfunctionalized crystal-like aggregates with gold-labeled streptavidin again resulted in marginal random deposition of gold particles (Figure 11B). In addition, it should be mentioned that the 2-D sheets of the biotin incorporated aggregates are significantly larger than the control unfunctionalized crystal-like aggregates, however the aspect ratio seemed not to be significantly affected. This accelerated growth in all directions is thus related to the incorporation of 4. Since these 2-D sheets are of a crystal-like nature, a possible explanation for
the accelerated growth in all directions can also be obtained using the analogy with crystal growth. The absence of the long alkyl tail in the molecular structure of 4 will result in a less tight molecular packing of the surfactant alkyl chains in the aggregate structure. As fast growing crystal planes are often thermodynamically less stable than the slower growing counterparts, a destabilizing effect in both the length and width direction of the aggregate upon incorporation of 4 can result in accelerated growth in all directions. This increase in general aggregate size was not observed for the ribbon aggregates, which might originate from the already present largely increased growth speed in the length direction as was discussed in chapter 5, which possibly overrules the effect of a less ordered molecular packing of the hydrophobic interior. However, for both the crystal-like and the ribbon-like morphology, addition of the biotin anchor moiety does not alter the aspect ratio.

6.4 Conclusion

It is demonstrated in this chapter that the bis-ureido based surfactant aggregates presented in the previous chapter could be functionalized via a modular approach through molecular recognition, with other bis-urea containing molecules. The selectivity of the molecular recognition was demonstrated through incorporation of the bis-urea functionalized azobenzene dye “disperse orange” within the aggregate structure. The UV-vis spectra of the resulting solutions showed distinct differences for both the matching and non-matching bis-urea functionalized dyes, which could be related to the binding efficiency of the two bis-urea units within the aggregate molecular packing. The observed large blue shift was ascribed to face-to-face H-type aggregation confirming the incorporation of the dye molecules within the ribbon structure and the immobilization of the chromophores at the aqueous interface.

Further functionalization of the surfactant aggregates was demonstrated for both the ribbons and the 2-D crystal-like morphologies by incorporation of biotin coupled to a matching bis-ureido group via a PEG-spacer. Selective decoration of the biotin functionalized aggregates with gold-labelled streptavidin, showed that biotin retained its biological functionality when incorporated into the aggregate structure and is accessible at the surface.

This proof of principle might be extended through modification of the molecules with short peptide fragments and eventually even growth factors with matching bis-ureido units, thereby creating a cationic surface that displays signaling molecules. Since the biotin modified aggregates could be deposited from an aqueous solution onto a substrate, this functionalization method would be ideal for the production of multilayer functionalized films containing DNA using the LbL technique. The incorporated spacer between the functionality and the bis-urea

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achor moiety can ensure that the bioactive group is exposed through the DNA top layer directing cell differentiation and growth, while maintaining the envisaged reduced inflammatory effect of DNA on the incoming cells.

6.5 Experimental

General materials and equipment. Compounds 2 and 3 were kindly provided by R. M. Versteegen and R. A. Koevoets; compound 4 was kindly provided by E. Wisse. The Dutch Spinoza Junior Researchers Institute supplied large quantities of 1, synthesized via the CDI route. 15 Nm and 25 nm gold-labeled streptavidin, incubation block buffer and 200 mesh carbon coated copper grids were purchased from Aurion. CTAB was purchased from Sigma-Aldrich. All solvents were purchased from Acros Chemica or Sigma-Aldrich and were of analytical quality. PBS buffer was prepared by dissolving 8 g (137mM) NaCl, 0.2 g (2.7 mM) KCl, 1.44 g (10 mM) Na2HPO4 and 0.24 g (2 mM) KH2PO4 in 800 mL demineralised water, after which the pH was adjusted to 7.4 using HCl or NaOH solutions and the total volume was brought up to 1L. Ultra pure water (Barnstead EASYpure LF system; R >17.7 MΩ-cm) was used for preparing the aggregates solutions. Transmission microscopy measurements were performed on a FEI Tecnai 20, type Sphera TEM operating at 200 kV with a LaB6 filament and a bottom mounted 1k x 1k Gatan CCD camera or on a JEOL JEM 2000 FX microscope equipped with a CeB6 filament operating at 80 kV. Images on the JEOL microscope were taken with a conventional bottom mounted 9 x 6.5 cm plate camera. UV-vis spectra were recorded on a Shimadzu Multispec-1501 equipped with a diode-array detector.

UV-vis experiments

Surfactant/dye mixtures. A 5 mg/mL (8.5 mmol/L) solution of 1 was prepared by dissolving this compound in chloroform/methanol (9:1 v/v) followed by heating to 60 °C for 5 min. and subsequent cooling. To this solution 6 mol% of 2 or 3 was added to obtain the surfactant/dye stock solutions. The UV-vis spectra of both stock solutions were recorded after dilution with chloroform to obtain a maximum absorption of 1 (46x for 2, 11x for 3). For the aqueous ribbon suspension, water was added to the undiluted stock solutions, followed by evaporation of the organic solvent while stirring vigorously to ensure proper mixing and to prevent boiling of the liquid. When all organic solvent was evaporated, the temperature was further increased to above 80 °C to ensure molecular mixing. When cooled to room temperature a yellow turbid suspension was reformed and water was added to obtain a final 5 mg/mL surfactant concentration. UV-vis spectra were recorded using the same dilution factor with water as was used for the chloroform stock solutions. The incorporation of 2 and 3 within CTAB micelles was achieved using the same procedure as described above, only starting with a 5 mg/mL CTAB solution in chloroform. Upon addition of water and evaporation of the organic solvent a clear orange solution was formed. Again the same dilution factor as mentioned above was applied for recording the UV-vis spectra.

Polarity study. Two stock solutions of 1.3 mg/mL dye (2 or 3) were prepared in 100% chloroform (stock A) and 100% methanol (stock B). To 25 μL of stock A, chloroform/methanol mixtures were added to a total volume of 500 μL to obtain the different polarity ratio’s. e.g. a 60% chloroform/methanol dye solution is obtained by adding 275 μL chloroform and 200 μL methanol to 25 μL stock A. Using this procedure a polarity range between chloroform and methanol was prepared with 100, 80, 60, 40, 20 and 0 v/v% chloroform/methanol mixtures. Similarly, a polarity range between 100% methanol and 10% methanol/water was prepared (100% water is impossible due to insolubility of 2 and 3 in water), only using 25 μL stock B as a basis for the polarity range, e.g. a 30% methanol/water mixture is
obtained by adding 125 µL methanol and 350 µL water to 25 µL stock B. Mixtures with 100, 90, 80, 70, 60, 50, 30, 10 v/v% methanol/water were prepared. No additional dilution was necessary to record the UV-vis spectra, however, all spectra depicted in this thesis were corrected for scattering.

**Transmission electron microscopy**

For the incorporation of bis-urea functionalized biotin (4) into the ribbon structure a 1 mg/mL (1.7 mM) and a 0.1 (0.17 mM) mg/mL solution of bis-urea surfactant 1 in chloroform/methanol (9:1 v/v) were prepared. 10 Mole% of 4 was added to the solution and the solution was mixed. Water was added and the organic solvent was evaporated while stirring vigorously. The temperature was increased further to above 80 °C and subsequent cooling to room temperature yielded a white viscous turbid suspension. For the deposition of the biotin functionalized ribbons on the TEM grid, a 200 mesh carbon coated grid was placed on a 20 μL droplet of the prepared ribbon suspension and left for 15 minutes. Subsequently, the grid was transferred to droplets of PBS buffer (pH 7.4) (2 times 5 min), block buffer (Aurion BSA-c™) (1 time 5 min) and again PBS buffer (2 time 5 min). Next, the grid was transferred to a streptavidin-gold solution (Aurion 25 or 15 nm gold particles), which was diluted 2 times with PBS buffer, for 60 min. This was followed by 6 wash steps on droplets of PBS for 5 min., followed by two wash steps on droplets of milli-Q water (2 times 5 min). Finally, the excess water was manually blotted using a filter paper, after which the grids were examined in the TEM. The same procedure was followed for the blank sample, i.e. the sample in which 4 was not mixed in. The 1 mg/mL ribbon suspensions were incubated with 25 nm gold conjugates and analyzed using a JEOL microscope, whereas the 0.1 mg/mL 2D crystal-like suspension were incubated with 15 nm gold conjugates and analyzed using the Sphera.

EDX measurements on the incubated biotin functionalized ribbons (1mg/mL), were performed using a FEI Tecnai 120 kV microscope in the STEM mode.

### 6.6 References


Chapter 6

DNA / Bis-Ureido based surfactant interaction –

A Langmuir monolayer study

Abstract

In this chapter we demonstrate that for the present system the general view on DNA-cationic surfactant Langmuir monolayers, in which DNA is bound directly underneath a closed monolayer surface, is only applicable when DNA is injected underneath a preformed monolayer. The most frequently applied method in the literature in which the surfactant molecules are spread and compressed on a DNA containing subphase does not result in the formation of a closed surfactant monolayer. Instead, although a fraction of molecules do form close packed islands, most surfactants form DNA-surfactant complexes at the air water interface, which prevent the formation of a homogenous closed monolayer. Evidence for these conclusions comes from Langmuir experiments and direct imaging of different DNA-surfactant monolayers using high resolution Cryo-TEM and tomography.
7.1 Introduction

Research on the interactions between cationic surfactants and DNA molecules is motivated predominantly by two possible medical applications: (i) the use of cationic surfactants as non-viral gene transfection agents (vectors)\textsuperscript{1-3} and (ii) as a means to adsorb DNA to a surface in DNA sensor devices (lab on a chip).\textsuperscript{4-6} Moreover, these studies are of interest because the condensation of DNA is an intriguing phenomenon in various biological processes,\textsuperscript{7-9} which are still not fully understood. Although DNA-surfactant complexes are also studied in the bulk, e.g. in the lamellar phase,\textsuperscript{10} the Langmuir-monolayer is a model system that is frequently used to study the interaction between DNA and cationic surfactants.\textsuperscript{11} The general model for such a system involves DNA molecules that are bound via electrostatic interactions underneath a closely packed monolayer of cationic surfactants. Experimentally, this arrangement can be approached via two different methods: (i) injection of DNA under a preformed, compressed monolayer and (ii) formation of a monolayer on top of a DNA containing subphase. As mentioned in chapter 3, the degree of interaction between DNA and surfactant can be assessed by observing changes in the surface pressure-surface area ($\Pi$-$A$) isotherm upon compression of the monolayer, in the presence or absence of DNA in the subphase. To date, however, few investigations related to the direct observation of the DNA morphology underneath a Langmuir monolayer without the use of intercalating dye’s are known.\textsuperscript{12}

![Figure 1. General model of DNA bound underneath a closely packed cationic surfactant monolayer at the air-water interface.](image)

The $\Pi$-$A$ isotherm of a cationic surfactant monolayer generally changes significantly when it is compressed on a DNA containing subphase instead of on a water subphase, however, this effect is also observed for other polyelectrolyte containing subphases.\textsuperscript{13-15} The change in the $\Pi$-$A$ isotherm, which is dependent on the DNA concentration,\textsuperscript{13} in general is characterized by an increase in the lift off area, a shift of the isotherm to higher mean molecular area and an increase in collapse pressure. This change in the isotherm has been attributed to the
formation and compression of a new DNA-surfactant complex possibly embedded between domains of the pure cationic surfactant.\textsuperscript{13,16,17} Brush-like structures have been suggested as a possible arrangement for the DNA-surfactant complexes,\textsuperscript{18} however hardly in conjunction with monolayers.\textsuperscript{19} Most cartoons on DNA-surfactant monolayers however show the image of DNA situated underneath a closely packed layer of surfactant molecules (Figure 1).\textsuperscript{1,12,20-22}

Brewster angle microscopy (BAM) has been used in the interpretation of Langmuir-isotherms,\textsuperscript{17,21,23,24} however, apart from a general surface morphology at a 1 μm resolution, no molecular information can be obtained with this technique. Other \textit{in situ} techniques like grazing incidence X-ray diffraction or infrared reflection absorption spectroscopy (IRRAS) can be employed to analyze the DNA-surfactant monolayer structure on a molecular level.\textsuperscript{21,22} For the molecular imaging of DNA, high resolution AFM can be employed, as was demonstrated by Yang et al.\textsuperscript{25} who studied the condensation of DNA on a preformed cationic lipid membrane, in which even the helical repeat of closely packed DNA chains could be observed. Both Langmuir-Blodgett and Langmuir-Schaefer transfer of a DNA-cationic surfactant monolayers at various surface pressures followed by subsequent analysis using high resolution AFM has been employed. Interestingly, none of these images showed DNA at a similar molecular resolution as found by Yang et al. but show different branched, fiber-like morphologies ca. 100 nm in width and several micrometers in length.\textsuperscript{12,13,16,17,19,21,23-32} This evokes an interesting question; i.e. why, in the case of a transferred DNA-surfactant monolayer, single DNA chains in a close packed arrangement are not observed, whereas they can be clearly distinguished on top of a preformed lipid membrane.\textsuperscript{25}

Since many years TEM has been employed to visualize individual DNA chains, however staining or chemical fixations are generally required. In 1994 using cryo-TEM on vitreous sections, Dubochet et al. succeeded in imaging individual DNA molecules in their native hydrated state,\textsuperscript{33} and even its superhelical structure could be observed. The same group later succeeded to image densely packed DNA in cryo-sections of human and stallion spermatozoa, with a chain stacking resolution of 2.7 nm.\textsuperscript{34,35}

In this chapter we report on the interaction between the bis-ureido based surfactant 1, presented in the previous chapters, and DNA as studied using Langmuir monolayers. During the proposed Layer-by-Layer self-assembly (chapter 8), DNA will interact with a highly ordered and dense cationic surface of surfactant molecules in the self-assembled ribbon structure and it is useful, therefore, to study this interaction in a model system. The response of the surfactant molecules on the presence of DNA molecules should give an indication of the stability of the ribbons upon DNA complexation. We use cryo-transmission electron microscopy (cryo-TEM) for the direct visualization with molecular resolution of DNA-
surfactant monolayers, which are prepared both by injection of DNA under a preformed monolayer and by formation of a monolayer on a DNA containing subphase. No evidence was found supporting the formation of a closed monolayer when the surfactant is spread on a DNA-containing subphase. When DNA was injected underneath a preformed surfactant monolayer, no penetration of DNA into the monolayer structure was observed. This strengthens the initial hypothesis proposed in chapter 5, that DNA chains are not able to penetrate a preformed bis-urea ribbon bilayer structure, a requirement for the nanometer scale separation of DNA/bis-urea surfactant aggregates in LbL assemblies.

7.2 Langmuir monolayers on water

Π-A isotherms were recorded for the bis-ureido based surfactant 1 spread on a water subphase (Figure 2A). Upon compression, no increase in surface pressure was detected until a molecular area of ~27 Å²/molecule was reached. At this point a sudden steep increase in pressure, indicative of a liquid condensed phase, was observed until a collapse was reached at a surface pressure of 56 mN/m. Brewster angle microscopy showed the presence of dense domains already at Π = 0 mN/m (Figure 2B). Upon compression to a MMA of 26 Å²/molecule, no increase in reflected light intensity for the regions in-between the domains was observed (Figure 2Ai). Rather, these domains merged upon further compression (Figure 2Aii). From the point were a continuous dense film was obtained a steep increase in surface pressure was observed. Extrapolation of the slope of the curve in this region to zero pressure represents the mean molecular area (MMA) of the individual molecules in the corresponding closely packed equilibrium state without external force.

The presence of dense domains at Π = 0 mN/m, indicates a strong self-assembling behavior of the surfactant molecules, which can be attributed to hydrogen bonding between the incorporated bis-urea groups. The size and shape of the domains stays the same up to the point where a continuous layer is formed. Consequently, the head group packing in the self-assembled islands is the same as the MMA deduced from the slope of the surface pressure increase. The deduced MMA of 21 Å²/molecule, corresponds very well to the MMA derived from the molecular model proposed for the ribbon aggregates. We conclude that the molecular organization in the monolayer is the same as in these ribbons (chapter 5).

* No liquid expanded phase, even at elevated temperatures up to 50 °C, could be observed in any of the recorded isotherms.
Figure 2. A. Surface pressure vs. surface area ($\Pi$–$A$) isotherms of bis-urea surfactant 1 spread and compressed on a water (—) and on a DNA containing (—–) subphase [DNA] = 3 mg/ml). The arrows indicate the MMA extrapolated to zero pressure. Inset: Brewster angle microscopy (BAM) images taken at various stages of compression as indicated by the roman numbers in the isotherm. B. BAM image of a dense domain with rough edges of surfactant 1 spread on a water subphase ($\Pi$ = 4 mN/m). C. Same as B but on a DNA containing subphase; note the smooth edge of the domain. D. BAM image of only the phase in-between the domains when the bis-urea surfactant is spread on a DNA containing subphase and compressed to a surface area of 26 Å²/molecule ($\Pi$ = 55 mN/m); note that the density at this surface pressure is still lower than that of the domains at 0 mN/m.

Spreading of the bis-urea surfactant on a DNA containing subphase (3 mg/ml) and subsequent compression yielded a completely different isotherm (Figure 2A). At the start of the
isotherm, zero pressure was not fully obtained indicating that the lift-off area was above 130 Å²/molecule. Upon compression a clear liquid expanded phase was observed from ~100 to 35 Å²/molecule. Below 35 Å²/molecule, the isotherm entered the liquid condensed state. The slope of the isotherm was clearly less steep than in the case of the liquid condensed phase on pure water, nevertheless, the maximum collapse pressure was significantly higher (66 mN/m). Extrapolation of the liquid condensed phase to zero pressure yielded a MMA of approximately 50 Å²/molecule, which is more than double the MMA of the surfactant on water. Brewster angle microscopy again showed domain formation already at low surface pressures (~ 4 mN/m), although the domains showed much smoother edges (Figure 2AIII/C) and appeared to be fewer in number. Upon compression, the domains were pushed together in a similar fashion as on pure water. However, in this case, the regions in-between the domains did show an increased density upon increasing the surface pressure (Figure 2D). This separate phase prevented the domains from fusing at high compression (Figure 2AIV). Judging from the extrapolated MMA (48 Å²/molecule), surfactant molecules cannot form a closed packed arrangement when spread on a DNA subphase.

![Figure 3](image_url)

**Figure 3.** A. DNA/EtBr solution dissolved and injected in water, underneath a bis-urea surfactant monolayer; the white arrow indicates the monolayer surface at the air-water interface and the double black arrow indicates the pink DNA solution at the bottom of the dish. B. Same as A only after 16 hours of mixing using a flow underneath the monolayer surface; notice that the pink DNA solution still did not reach the monolayer surface. C. Same as A with the DNA/EtBr solution dissolved in water and injected in PBS buffer; notice that the pink DNA solution immediately attaches to the monolayer surface. D. Same as C only after 10 minutes mixing; notice that the pink colour is homogenously distributed throughout the complete solution.

The injection of DNA underneath a compressed monolayer that was kept at a constant pressure of 35 mN/m on a water subphase did not result in any change in surface area. To study if binding of the DNA molecules to the monolayer occurs, a DNA solution in water was
stained using the intercalating dye ethidium bromide (EtBr) and injected in the water subphase underneath a preformed bis-urea surfactant monolayer in a glass dish. Surprisingly, independent of the DNA concentration (between 5 and 30 mg/ml), the injected solution sank to the bottom of the dish and diffusion of the pink DNA containing phase hardly occurred (Figure 3A), even when a circulating flow underneath the monolayer was applied for 16 hrs. (Figure 3B). The same effect was observed when water was replaced by phosphate buffer saline (PBS, pH 7.4), both in the injected solution as in the subphase. A possible explanation for this behavior might be found in the higher ionic strength and the related density of the injected solution. Indeed, when the water subphase was replaced by PBS, injection of DNA dissolved in pure water did result in a rapid mixing of DNA solution and subphase (Figure 3C) and was complete within minutes (Figure 3D).

7.3 Langmuir monolayers on buffer solutions

In order to compare the situations in which DNA is injected or already present in the subphase, the Π-A isotherms were repeated using PBS as the subphase. The isotherm of the bis-urea surfactant on PBS showed a higher lift-off area (50 Å²/molecule) when compared to the experiments performed on water (Figure 4), however, the onset of the liquid condensed state was still observed at 25 Å²/molecule. The maximum collapse pressure was somewhat lower (52 mN/m) and the slope of the curve in the liquid condensed phase was slightly less steep. Nevertheless, the extrapolated MMA at zero pressure still amounted to 22 Å²/molecule indicating a densely packed film. Also the isotherm for the surfactant spread on a DNA containing PBS subphase (3 mg/ml) reached a lower maximum collapse pressure (58 mN/m) and showed a less steep increase in surface pressure upon compression. Extrapolation to zero pressure of the liquid condensed region yielded a slightly lower molecular area of 44 Å²/molecule, again approximately two times the MMA found for the surfactant on buffer alone. Due to screening of the charges caused by pH control and high salt content, the lower repulsion between the charged head groups will result in a lower surface pressure, which explains the observed decrease for the surfactant on a DNA/PBS subphase. Nevertheless, it is also known that highly charged surfactants have a tendency to oppose phase transitions and thus screening of the charge may result in their appearance.
The observed early lift-off and subsequent small increase in pressure before the liquid condensed phase for the surfactant on water can be the result of this effect.\textsuperscript{37-39}

When the Π-A isotherm of a 3 mg/ml DNA subphase was recorded without a surfactant present (Figure 4, inset), a small increase in surface pressure was observed upon compression indicating the surface activity of DNA molecules. However, addition of the isotherms of DNA and of surfactant on buffer did not yield the recorded Π-A isotherm of the surfactant on a DNA containing subphase, suggesting that the surface activity of DNA alone does account for the observed large liquid expanded region. This again supports the idea that a new separate phase is formed when the bis-urea surfactant is spread on a DNA containing subphase.

Injection of an aqueous DNA solution in a PBS subphase underneath a preformed monolayer kept at a constant pressure of 35 mN/m again did not result in any change in surface area. In this experiment the DNA solution was completely mixed through the subphase by 10

**Figure 4.** Surface pressure vs. surface area isotherms (Π-A) of bis-urea surfactant spread and compressed on a PBS buffer (---) and on 3 mg/ml DNA/PBS subphase (----). The arrows indicate the extrapolated MMA at zero pressure. Inset shows Π-A isotherms of the compression of surfactant on PBS buffer (a)(····), surfactant on 3mg/ml DNA/PBS subphase (b)(---), a 3 mg/ml DNA subphase (c)(---) and the sum isotherm of a and c (d) (····), versus the surface of the trough.
minutes flow circulation.† These results confirm that in this configuration DNA does not penetrate the monolayer structure and may form a complex as outlined in figure 1.

7.4 Cryo-TEM on DNA-Surfactant monolayers

![Image: Vitrobot environmental chamber](image)

**Figure 5.** The Vitrobot fitted with a humidity and temperature controlled glove box, in which a glass dish (depicted in figure 3) is placed. The schematic representation of the dish shows a surfactant monolayer at the air-water interface. A Quantifoil® holey carbon grid is placed underneath the monolayer prior to spreading of the surfactant. A DNA solution can be injected into the circulating flow. DNA is injected before or after surfactant spreading, after which the monolayer can be lowered on top of the grid using a drain mechanism.

To directly image the surfactant and DNA-surfactant monolayers using cryo-TEM a method, first reported by Leiserowitz et al., was adapted (Figure 5). A self-organized monolayer of bis-urea surfactant molecules was formed on top of a PBS subphase in a glass dish, with a density corresponding to a surface pressure of ~35 mN/m. The dish was situated inside a humidity and temperature controlled glove box and placed underneath a fully

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† In a separate experiment the mixing was confirmed by using ethidium bromide.
automated vitrification robot. Before spreading of the surfactant, a TEM grid supporting a holey carbon film was placed on a stainless steel mesh submerged underneath the air-water interface. During spreading on PBS buffer, the formation of a rigid film on top of the water surface could be detected by eye, which was allowed to equilibrate for 10 minutes. Subsequently, while maintaining 100% humidity to prevent evaporation, the formed monolayer was lowered onto the carbon grid using a drain mechanism. Inside the glove box, the grid was transferred to the plunging mechanism and raised into the environmentally controlled chamber of the Vitrobot. Next, the grid was blotted and vitrified by plunging into liquid ethane, after which it was analyzed using low dose cryo-TEM at -170 °C. The recorded low-magnification images of the surfactant monolayer on buffer showed a dense layer, with a patch-like structure covering the grid analogous to the layer of fused domains observed in Brewster angle microscopy (Figure 6A).

A similar procedure was used for the DNA-surfactant monolayers, where an aqueous DNA solution was injected in the PBS buffer subphase and mixed for 10 minutes using flow circulation. The injected DNA concentration was chosen such that the final subphase concentration amounted to 3 mg/ml, equal to the Langmuir experiments. The bis-urea surfactant was spread either before or after injection of the DNA solution and allowed to settle for 10 minutes, after which the subphase was drained and the DNA-surfactant monolayer was lowered over the TEM grid.

Low magnification images of the DNA-surfactant monolayer prepared by injection, showed a similar patch like structure as was observed for the pure surfactant (Figure 6B), whereas the surfactant spread on a DNA subphase did not show the formation of these structural features. In stead, these showed formation of large isolated dark domains in-between regions composed of a less dense DNA-containing phase (Figure 6C), which further confirms the observation that no closed monolayer is formed when the surfactant is spread on top of a DNA subphase.

High-resolution cryo-TEM images of the recorded DNA-surfactant monolayer in both cases showed the presence of many “wormlike” structures with diameters of 2.7 ± 1 nm, corresponding to the dimensions of DNA (Figure 7). The micrographs of the injected DNA sample underneath a preformed monolayer, showed a remarkably dense surface coverage of DNA chains (Figure 7A). The apparent inter-chain distance in projection was estimated to be 5 ± 2 nanometer.

Cryo-TEM images from a vitrified sample of a 3 mg/ml DNA solution without an applied monolayer clearly showed individual DNA chains, however with an average estimated apparent inter-chain distance in projection in the order of 40 nm (Figure 7C). Comparison of
these values implies that when injected underneath a preformed monolayer, there is a significant concentration of DNA chains at the air water interface, in line with the general model of a DNA-surfactant monolayer. The apparent inter-chain distance observed in a 50 mg/ml DNA sample was estimated to be $10 \pm 2$ nanometer (Figure 7D), which is still less densely packed compared to the DNA assembled underneath the surfactant monolayer.

**Figure 6.** A. Low magnification cryo-TEM image of a compressed surfactant monolayer on PBS buffer calculated for $\Pi \sim 35$ mN/m, with arrows showing cracks at which the domains observed in BAM are fused. B. Same as A, only with DNA injected underneath, with arrows indicating similar cracks as observed in A. C. Same as A only for surfactant spread on a DNA subphase, with arrows showing isolated dark patches on top of or in-between a DNA rich phase.
In contrast, the micrographs of the monolayer formed on top of a DNA containing subphase did not show a similar concentration effect. In this case, the density of DNA chains appeared to be much lower displaying an estimated apparent inter-chain distance in projection in the order of 20 nm (Figure 7B) and it was possible to still distinguish individual DNA chains.

Figure 7. A. Cryo-TEM image of DNA injected underneath a preformed bis-urea surfactant monolayer. B. Cryo-TEM image of a DNA surfactant monolayer, in which the surfactant molecules are spread on a DNA subphase. The darker area on the lower right part of the image represents the edge of a patch, similar as that observed in figure 6C. C. Cryo-TEM image of a 3 mg/mL DNA solution. D. Same as C only for a concentrated 50 mg/mL DNA solution.

7.5 Cryo-TEM tomography on DNA-Surfactant monolayers

Although the concentration effect for the injected DNA solution is evident, the transmission images do not show whether these molecules are actually bound to the monolayer at the air-
water interface. Using Low–dose cryo-tomography a tilt series from -70° to + 70° was recorded for the injected DNA-surfactant monolayer and subsequently reconstructed to a 3-D volume.

**Figure 8.** Reconstruction of a cryo-section of a DNA surfactant monolayer, in which DNA was injected underneath a preformed bis-urea surfactant monolayer. **A.** Projection along the x- and y-direction of the 3-D reconstructed volume in reverse contrast showing the 2.5 nm DNA strands suspended from the monolayer. Parts of the DNA strands are attached to the cationic monolayer surface resulting in a thin dense layer of DNA at the monolayer surface (indicated by the white bar), while the other ends are extending down into the bulk solution. **B.** Unprocessed x-, y-, and z-cross-sections combined to form a 3-D volume. The horizontal z-cross-section is positioned at the monolayer surface (indicated by the black arrows) and clearly shows the presence of many worm-like structures in the horizontal plane. **C.** Same as **B** with the horizontal cross-section positioned below the monolayer surface. Only a few DNA strands are present when compared to **B.** **D.** Same as **C** only with the horizontal cross-section positioned lower, showing a similar image as **C.**

Although the concentration effect for the injected DNA solution is evident, the transmission images do not show whether these molecules are actually bound to the monolayer at the air-water interface. Using Low–dose cryo-tomography a tilt series from -70° to + 70° was recorded for the injected DNA-surfactant monolayer and subsequently
reconstructed to a 3-D volume. Vertical cross-sections in the x- and y- direction of the reconstructed volume revealed a 10 ± 3 nm layer of dense matter present at the top of the vitreous section, i.e. at the ice-vacuum interface. Horizontal cross-sections (z-slices) positioned at this layer showed the presence of many worm-like structures (Figure 8B). When the z-slice is positioned anywhere below the dense 10 nm layer, the chain density is significantly decreased and individual DNA strands can be clearly distinguished (Figure 8C/D) comparable to the 3 mg/ml DNA solution in bulk. The information from the transmission images of densely packed DNA chains in section 7.4 of this chapter, originates predominantly from the layer of condensed matter in the top ~10 nm of the vitrified ice layer, i.e. from the air-water interface.

A side view of the total reconstructed 3-D volume clearly shows that the DNA strands are suspended form the surface down into the subphase (Figure 8A). Apparently, the individual DNA strands do not bind completely to the monolayer surface and a part of the chain extends down into the subphase. Apart from the superposition of all DNA chains present in the total section, this may also explain why complete DNA strands (end-to-end) are not observed in the transmission image presented in figure 7A).

7.6 Conclusion.

When surfactant 1 is spread on a water or buffer subphase, domains are formed that can be compressed up to the point of fusing into a single continuous layer. The head group distance of the surfactant molecules within this monolayer matches that of the ribbon aggregates of 1. When DNA is injected in a buffer subphase underneath a preformed monolayer of 1, DNA binds directly underneath the dense monolayer surface as was apparent from the Cryo-TEM and tomography images and does not penetrate the monolayer structure as could be deduced from the zero surface area change in the II-A isotherm upon DNA injection. This points to the general model proposed for DNA-surfactant Langmuir films. However, it should also be noted that most DNA chains do not bind completely to the monolayer surface, but have a part of the strand extending into the sub-phase. Nevertheless, it can be assumed that a preformed ribbon surface, which has the same molecular packing as the monolayer surface, will also not be penetrated by DNA molecules when using the Layer-by-Layer deposition technique in conjunction with DNA.

When DNA is present in the subphase, spreading of the surfactant still leads to domain formation, as was evident from BAM and the dark patches in low magnification TEM images, however, these are prevented to fuse into a compact monolayer surface by a DNA containing phase, which is situated between the domains. The summation of the isotherms of pure DNA
and pure surfactant does not result in a similar isotherm as observed for the surfactant spread on top of a DNA subphase. The DNA rich phase, which passes through the liquid expanded state, can therefore not be composed of DNA alone and is most likely an aggregate of DNA and surfactant. The type or shape of the aggregates that are formed cannot be determined from the data presented here, although brush-like structures of DNA and cationic surfactants are likely due to the ionic interaction of the head group of the surfactant with the outside of the DNA helix.

In light of the results presented above, it can now be explained why the results in chapter 3 using the polymerizable surfactant on a DNA subphase did not yield the expected results. Also in this case, significant changes, even larger than for the bis-urea surfactant, were observed when comparing the isotherms of the polymerizable surfactant spread on water and spread on a DNA containing subphase. Most likely, a closely packed surfactant monolayer was not formed in this case. If DNA-surfactant complexes were formed instead, compression and polymerization of these structures would result in mixing two component phases having both a hydrophilic (DNA and surfactant head group) and hydrophobic (surfactant alky tails) character, held together by covalent bonds and ionic interactions. This would explain the unexpected high transfer efficiency to hydrophilic substrates and also why the DNA was not positioned on the outside, but rather on the inside of the transferred monolayers.

Finally, it can be speculated that if the shape of the recorded isotherms, when comparing the surfactant spread on water or spread on a DNA subphase, changes significantly, a different complex phase that does not necessarily correspond to the general model has been formed.

7.7 Experimental.

General materials and equipment. Bis-urea surfactant 1 was supplied by The Dutch Spinoza Junior Researchers Institute. DNA (~300 bp/molecule; sodium salt) was kindly provided by Nichiro Corporation (Yokosuka-shi, Kanagawa prefecture, Japan). Potential protein impurities in the DNA were checked using the BCA protein assay (Pierce, Rockford, Illinois, USA) and measured to be below 0.20% w/w. Ethidium Bromide was purchased from Bio-Rad. All solvents were purchased from Acros Chemica or Sigma-Aldrich and of p.a. quality. R2/2 Quantifoil Jena grids were purchased from Aurion. PBS buffer was prepared by dissolving 8 g (137mM) NaCl, 0.2 g (2.7 mM) KCl, 1.44 g (10 mM) Na2HPO4 and 0.24 g (2 mM) KH2PO4 in 800 mL demineralised water, after which the pH was adjusted to 7.4 using HCl or NaOH solutions and the total volume was brought up to 1L. Ultra pure water was used in all Langmuir experiments (Barnstead EASYpure LF system; R >17.7 MΩ·cm). The Langmuir isotherms were recorded using a KSV 3000 computer controlled system equipped with a temperature controlled removable Minitrough (75.0 x 323 mm) constructed from a solid piece of PTFE. The hydrophilic barriers are were made from polyacetal (Delrin). The surface pressure was recorded using a filter paper (10 x 20 mm) (the bis-urea surfactant}
adsorbs platinum of the Wilhelmy plate). The complete KSV 3000 system was situated in a sealed polycarbonate cabinet. Brewster angle microscopy measurements were performed on a KSV Minitrough system equipped with a BAM 300 microscope, which uses a HeNe-laser (10 W, 633 nm) with Glan-Thompson polarizers (10-8 polarization ratio) and a computer controlled high definition CCD camera (768 x 576 pixels). Transmission microscopy measurements were performed on a FEI Titan Kryos TEM equipped with a field emission gun (FEG) operating at 300 kV. Images were recorded using a Gatan GIF energy filter and a 2k x 2k Gatan CCD camera. A Gatan cryoholder operating at ~ -170 °C was used for the cryo-TEM measurements. The sample vitrification procedure was carried out using an automated vitrification robot, viz. a FEI Vitrobot™ Mark III equipped with a humidity and temperature controlled glove box. The Quantifoil grids were treated with a surface plasma treatment using a Cressington 208 carbon coater operating at 5 mA for 40 seconds prior to the sample preparation and vitrification procedure. The tomography reconstruction was performed using the Inspect3D (FEI company) software program version 2.1. Additional image analysis was performed using Amira version 3.1.1. Extreme care was taken that all used equipment related to monolayer experiments was thoroughly cleaned.

**Langmuir experiments.** A 1 mg/mL chloroform solution of bis-urea surfactant 1 was prepared by accurately weighing 10 mg of 1 into a closed vial and adding of 8 mL of chloroform, followed by heating to 60 °C and sonication for 5 min. to ensure complete dissolution. After cooling to room temperature, the solution was transferred quantitatively to a 10 mL volumetric flask and brought up to volume. In general 25 μL was spread unless indicated otherwise. The surfactant was spread on either water, PBS buffer or a 3 mg/ml DNA solution dissolved either water or PBS, after which the system was compressed with a 5 mm/min compression rate. The isotherms of a DNA solution injected underneath a compressed monolayer were recorded by spreading 35 μL on top of a water or PBS subphase. Subsequently, the system was compressed to and maintained at a surface pressure of 35 mN/m. A peristaltic pump was used to create a circular flow of DNA underneath the monolayer surface (rate: 8 ml/min), with each end of the tubing, which contained a DNA solution (calculated to a total DNA subphase concentration of 3 mg/mL), positioned underneath one of the barriers. A stained DNA solution with Ethidium Bromide showed complete homogeneous mixing of the pink colour throughout the subphase in the case of a PBS subphase. When the pink solution reached the monolayer surface no significant change in MMA could be observed at constant surface pressure.

**BAM measurements.** Brewster angle microscopy measurements were performed on monolayers of surfactant 1 spread on a water and spread on a 3 mg/mL DNA subphase in water. The experiment were performed using a KSV Minitrough system equipped with a BAM 300 microscope following the same procedure as described above. During compression a BAM image was recorded every 10 seconds, after which the image was coupled by the BAM software to the corresponding MMA of the recorded isotherm.

**DNA subphase mixing experiments.** To investigate the mixing of an injected DNA solution underneath a preformed monolayer a glass dish was constructed with a closed circuit connection to a peristaltic pump at either side of the dish. Surfactant 1 was spread from a 1 mg/mL chloroform solution on a water of PBS subphase calculated to have a surface pressure of 35 mN/m. During subphase mixing (8 mL/min) the DNA solution was slowly injected into the tubing of the peristaltic pump. The injected DNA solutions were prepared from water to a total concentration of 5, 10 and 30 mg/mL DNA and thoroughly mixed with 200 μL of a concentrated 10 mg/mL Ethidium Bromide solution. The amount of injected volume was calculated such that the final subphase DNA concentration amounted to 3 mg/mL and the total volume of subphase, injected DNA and tubing did not cause the dish to overflow. Images were taken at different time intervals after injection using a standard digital camera.
Transmission electron microscopy

Conventional cryo-electron microscopy. The cryo-TEM images of the 3 and 50 mg/mL DNA solutions were obtained by applying small aliquots (3 μl) of the viscous solutions to Quantifoil grids (R2/2 Quantifoil Jena) within the environmental chamber (relative humidity 100%) of the Vitrobot™ at 22 °C. Excess liquid was blotted away with filter paper using an automatic blotting device within the environmental chamber of the Vitrobot™. The grid was subsequently shot through a shutter into melting ethane placed just outside the environmental chamber. The vitrified specimens were stored under liquid nitrogen and observed at -170 °C (Gatan cryo holder) in the Titan microscope. Micrographs were taken at 300 kV using a GIF energy filter at low-dose conditions.

Monolayer cryo-electron microscopy. The glass dish, as described in the above DNA mixing experiments, was placed in a temperature a humidity controlled glove box underneath the environmental chamber of the Vitrobot. The dish, and connected tubing of the circular peristaltic pump (with a total volume of 54 ml) were filled with 40 mL PBS buffer and air was removed from the system. A bend mesh was placed below the air-water interface and hydrophilic Quantifoil grids were placed on top. Care was taken that the tubing connecters and the grid were situated well below the water line. A 1mg/mL chloroform solution of surfactant 1 was spread calculated to a total surface pressure of 35 mN/m and allowed to equilibrate for 10 min. During mixing (8 ml/min), 13 ml of a 11.5 mg/ml DNA solution in water was slowly injected into the tubing. After injection flow circulation was continued for 10 min. to ensure complete mixing of the DNA with the subphase solution, after which the monolayer was slowly lowered onto the Quantifoil grid using a draining tube at the bottom of the dish. While at 100% humidity, the grid was attached to the plunger mechanism and raised into the environmental chamber of the Vitrobot. Subsequently, the glove box was removed and replaced by a tray containing liquid ethane at its melting temperature (-170 °C). After blotting the grid was shot through a shutter into the melting ethane. The vitrified specimens were stored under liquid nitrogen and observed at -170 °C (Gatan cryo holder) in the Titan microscope. Micrographs were taken at 300 kV using a GIF energy filter at low-dose conditions.

For the monolayer spread on a DNA subphase the same procedure was followed with the exception that the DNA was injected and mixed during 10 min. before the bis-urea surfactant was spread. After spreading on the DNA subphase the monolayer was also allowed to equilibrate for 10 min. after which the draining and vitrification procedure was started.

Cryo-TEM tomography. The samples prepared in the above section were also used for low-dose cryo-TEM tomography; 300 μl of anionic gold tracer solution (15 nm gold particle size, Aurion) was added to the injected DNA solution for feature tracking during the reconstruction procedure. The region of interest was selected and placed in optimal position for a maximal tilt angle to both sides. The tilt series was recorded on a Titan microscope operating at 300 kV using a GIF energy filter together with the full range of the 2k x 2k CCD camera and low-dose conditions. The Xplore3D software was used to acquire the tilt series.

7.8 References


DNA-Surfactant Layer-by-Layer coatings –

Analysis of a layered system

Abstract

Here the concepts presented in the previous chapters are applied to produce a truly layered DNA-based coating, in which the individual layers are separated on a nanometer scale. The bis-ureido surfactant bilayer aggregates are used to replace the cationic polymer component in the Layer-by-Layer deposition of DNA-based films. It is demonstrated that during the deposition from aqueous solutions, the ribbon aggregates and DNA chains do not mix, thereby resulting in the formation of a layered structure with nanometer dimensions. Functionalization of the surfactant aggregates with biotin as described in chapter 6 resulted in the selective incorporation of this bio-molecule in one individual layer, as was shown after binding of gold-labeled streptavidin, opening up possibilities for surface signaling and compartmentalization. It was found that the LbL deposition efficiency is largely determined by the adhesion of the ribbon aggregates to the underlying substrate or DNA terminated layer and that the aggregate layers are not completely closed.
8.1 Introduction

In the previous chapters of this thesis we have introduced the Layer-by-Layer technique as the most suitable coating technique for the production of multi-layered DNA based biomaterial coatings.\footnote{1} In principle, the alternate absorption of negatively charged DNA and a cationic polymer component should yield a layered structure allowing the incorporation of medicine or growth factors in individual layers for controlled drug delivery and cell signalling.\footnote{2-18} In chapter 4, it was demonstrated that PDL and PAH could be used as the cationic polymer components to construct DNA-based LbL coatings\footnote{19} and that these coatings could be enriched with growth factors such as BMP-2. It was further demonstrated that these films were cyto- and histocompatible and induced an increase in cell proliferation.\footnote{20} As most polymer based LbL films, the PAH/DNA and PDL/DNA films showed a mixed surface, which indicated that no truly layered structure on a nanometer scale was formed. This is a consequence of the fact that the polymer chains are able to diffuse throughout the film, with higher diffusion rates leading to increased mixing and exponentially growing films.\footnote{16,21-25} It has been demonstrated that even linearly growing films, which are characterized by limited diffusion of the constituting components, show a layer thickness in the order of hundreds of nanometers still pointing to an appreciable degree of diffusion. This mixed layer structure has a few limitations with respect to the application of these films as a biomaterials coating. Firstly, it is difficult to ascribe the promising \textit{in vitro} and \textit{in vivo} results purely to the DNA, as both the anionic and cationic components are present at the coating surface. Secondly, the absence of discrete layers does not allow the incorporation of drugs or growth factors in specific predetermined layers such that a sequential release of different components can be orchestrated. In literature, attempts to solve this problem have resulted in a combined coating of linear and exponential growing films, in which the linear growing multilayers act as barriers between exponentially growing multilayers,\footnote{16,17} thereby creating a multi-compartment coating. To prepare DNA based coatings layered on a molecular level we decided to replace the cationic polymer with a component that does not mix with DNA. Inspired by the lamellar phase of DNA-surfactant complexes,\footnote{26} we used an ammonium bis-ureido surfactant (1), which forms well-defined highly ordered bilayer aggregates in water. As described in chapter 5, the size and shape of these aggregates can be tuned by using specific growth conditions. In the previous chapter it was demonstrated that DNA was not able to penetrate a preformed monolayer assembly of surfactant 1. From this it was anticipated that, when using the LbL deposition technique in conjunction with DNA, diffusion of the DNA into these stable, densely packed ribbons would be largely reduced. In addition, it was demonstrated (chapter 6) that these aggregates could be
functionalized in a modular approach through molecular recognition of other bis-urea containing molecules,\textsuperscript{27} which opens up possibilities to anchor e.g. growth factors in individual layers as well.

In this chapter we demonstrate the formation of a DNA-based coating with a nanometer scale layered structure using the LbL deposition of bilayer aggregates of \textit{I} and DNA (Figure 1). In addition the first \textit{in vitro} tests of these coatings are presented.

\textbf{Figure 1.} Schematic changes in DNA-based LbL coating, with (left) a mixed polymer based system (dots represent additives) and (right) a truly layered multi-compartment structure.

\section*{8.2 Coating construction}

Multilayer [ribbon/DNA] films were first deposited on quartz substrates, using alternatingly an aqueous 1 mg/ml suspension of \textit{I} and an aqueous 1 mg/ml DNA solution, with an immersion time of 7 minutes followed by a 3 minute wash step and drying using a stream of nitrogen. It has been demonstrated that the first deposited layer is important for an effective build-up of the film.\textsuperscript{28} Therefore, in analogy to the previously described polymer based DNA LbL coatings (see chapter 4), the first ribbon layer was deposited using a dipping time of 30 minutes in a concentrated 5 mg/ml ribbon suspension, followed by a 3 minute wash step (method 1). The build up of 10 double-layers was monitored using the stepwise increase of the characteristic 260 nm DNA UV-absorption (Figure 2A). After each successive layer of DNA, the 260 nm UV absorption showed a linear increase in intensity (Figure 2B), which indicated that an equal amount of DNA had been deposited with each additional step. The total optical density (O.D.) after 10 successive double layers amounted to 0.10, with an average increase of 0.01 per deposited layer of DNA. The initial DNA absorption (O.D. \textasciitilde 0.01, corrected for scattering) was in agreement with the successive cumulative increase of the following layers showing that the amount of DNA deposited after the first ribbon layer was equal to that deposited in the following layers.
Figure 2. A. Increase in 260 nm DNA UV-absorption measured after each added layer of DNA and corrected for the increase in scattering due to the previously absorbed ribbon layer. B. Corrected increase in the intensity of the 260 nm absorption band plotted versus the number of double-layers (DNA-terminated), showing a linear increase.

Figure 3. Corrected cumulative 260 nm UV-absorption for method 1 and 2 as a first layer deposition method showing a similar linear increase; however, an initial burst absorption for method 2. Dotted line indicates the initial 260 nm absorption after the first deposited DNA layer; the cumulative increase above this line is similar for both methods.

To investigate whether the build-up efficiency would improve when a thicker first ribbon layer was deposited, a 1 mg/ml ribbon solution was drop cast without a subsequent washing step as first layer deposition (method 2). The following layers were deposited as described above. The 260 nm DNA UV-absorption showed a linear cumulative increase to a total absorption of ~0.16 and an average increase of ~0.01 per double layer, indicating no significant improvement in build-up efficiency for the following double layers. However, the initial absorption of the first DNA layer appeared to be significantly higher for method 2 (O.D. = ~0.06, corrected for scattering) compared to method 1 (O.D. = ~0.01) (Figure 3). It is therefore
speculated that a drop cast first layer without a subsequent washing step (method 2) results in the deposition of more ribbon material when compared to method 1 and that subsequently also more DNA is absorbed. TEM indeed confirmed that washing (3 min. continuous flow) of a drop cast ribbon layer (Figure 4A) results in the removal of many of the deposited ribbon aggregates (figure 4B). In contrast, the following absorbed aggregate layers are similar for both methods since they both rely on adsorption of the aggregates from solution onto the coating surface and are both followed by a subsequent washing step, which results in an equal amount of deposited ribbons and therefore also an equal amount of deposited DNA. A thicker first deposited layer, therefore, had no effect on the total build-up efficiency.

![Figure 4](image)

**Figure 4.** Silicon based TEM grid with deposited ribbons using the drop cast method 2. A. Prior to a 3 minute wash step. B. After washing, showing a significant decrease in deposited aggregates.

### 8.3 Coating topology

To verify whether the DNA and ribbon aggregate layers are truly separated, the surface topology of the multilayer films was analyzed using atomic force microscopy (AFM) after the deposition of each individual layer up to 6 double-layers using method 1. For ribbon-terminated layers, the contours of individual ribbons could be clearly distinguished (Figure 5A), whereas DNA-terminated layers showed contours of a patch-like topology (Figure 5B). In addition, small height differences originating from overlapping ribbons of the underlying layer were superimposed onto the DNA patches and vice versa. To determine which structure is on top the amplitude images were assessed.
Figure 5. Characteristic surface topology measured using AFM. A. Height image of a ribbon terminated layer showing ribbons covering the surface. B. Same as A only DNA terminated showing a patch-like morphology. C. Amplitude image of A. D. Amplitude image of B.

Features that are present at the surface appear more “sharp” in the amplitude image compared to features originating from underlying structures, which are dampened. In the amplitude image it can be clearly seen that the ribbons appear more “sharp” on ribbon terminated layers (Figure 5C) and that DNA patches are more pronounced on the DNA terminated layers (Figure 5D). Closer examination of the ribbon terminated layers showed that most parts of the substrate were covered, however also areas in which the underlying DNA layer was exposed could be observed. DNA terminated layers showed patches covering the entire surface indicating that the DNA layers are more closed than the ribbon layers.
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Figure 6. A. TEM image of a 5 double-layer film of DNA and crystal-like aggregates of surfactant 1. B. AFM image of A showing that the patches are covering the surface.

These results indeed confirm that during the LbL process the DNA and ribbon aggregates do not mix and form separate layers, although the formed ribbon layers are not completely closed.

TEM on a LbL film composed of DNA and aggregates of the 2-D crystal-like morphology (see chapter 5) deposited on a carbon coated copper grid, also showed a similar patch-like morphology present throughout the complete transmission image (Figure 6A). It should be noted that these patches are smaller than those observed in figure 5, however, the washing steps for the TEM grids were less intensive than those for coatings deposited on glass slides. AFM measurements on these TEM samples confirmed that these patches are covering the surface on DNA terminated films (Figure 6B). Also TEM images showed areas, which were not covered with surfactant aggregates, but did show the continuing presence of the patch-like morphology* confirming that the DNA layers are fully closed whereas the aggregate layers are not.

* DNA can also adsorb to the underlying carbon support film.
8.4 Spectral analysis.

Since AFM and TEM can only probe limited surface areas up to the micrometer regime, X-ray photoelectron spectroscopy (XPS) was employed to analyse the coating surface on a millimeter scale. The phosphorus/nitrogen ratio (IP$_{2p}$/IN$_{1s}$) was determined for all recorded spectra measured for each sequential layer up to 4 double layers. The spectra were recorded at both 0° and 60° with respect to the surface normal. As already mentioned in chapter 4, the penetration depth at 0° is about 10 nm, whereas at 60° it is limited to approximately 5 nm. Since the ribbon aggregate thickness amounts to 6 nm, no phosphorus signal is expected for ribbon terminated layers when measuring at a 60° angle in the ideal situation i.e. when the layers are closed and truly separated. In contrast, DNA terminated layers should show a relatively larger IP$_{2p}$/IN$_{1s}$ ratio for the 60° signal compared to the one recorded at 0° due to enrichment in phosphorus in the top 3 nm (DNA chain thickness ~2.5 nm).

Surprisingly, all measured layers, even the ribbon terminated ones, showed a higher IP$_{2p}$/IN$_{1s}$ ratio for the 60° measurement (Figure 7), indicating that the DNA responsible for the measured phosphorus signal always constitutes the top layer. This further implies that the surface contains DNA patches that are not covered with ribbons also in the ribbon terminated layers.

The theoretical value of the IP$_{2p}$/IN$_{1s}$ ratio measured at 0° of a closed and truly layered film, in which the top 3 nm consists of pure DNA and the underlying 6 nm of ribbon aggregates, can be estimated to be ~0.1.$^\dagger$ The IP$_{2p}$/IN$_{1s}$ ratio of DNA terminated layers converges to this theoretical value and amounts to 0.075 after the deposition of 10 double layers (data not shown). This trend is even more pronounced when the washing step in-between the dipping steps is skipped (Figure 7). As can be expected for incomplete coverage surface coverage, the IP$_{2p}$/IN$_{1s}$ ratio decreases only moderately for the ribbon terminated layers, whereas for complete aggregate coverage it should decrease approximately to zero since only 10% of the total signal originates from a depth below 6 nm (theoretic IP$_{2p}$/IN$_{1s}$: ~0.02).

The XPS results confirmed the AFM and TEM measurements also on a millimeter level: the ribbon layers are not fully closed, however, they do form separate layers with DNA, as judged from the observed angle dependence for phosphorus. Since DNA does form closed layers and can only be detected when it forms the top layer, this entails that on DNA terminated layers the surface is exclusively covered with DNA and that the observed biological

$^\dagger$ The measured IP$_{2p}$/IN$_{1s}$ ratio of a drop cast DNA layer is ~0.2. Based on the escape probability of an electron from a polymer film, ~50% of the measured signal originates from the top 3 nm of the film, and ~90% from the top 6 nm.
effects on the coating presented in the following section, can be ascribed to the presence of DNA.

![Graph showing phosphorus/nitrogen ratios](image)

**Figure 7.** XPS data showing the phosphorus/nitrogen ratios measured at 0° (—) and 60° (—–) for the samples prepared on glass substrates and at 0° for samples prepared without a washing step in-between the dipping steps (— - -). The even numbers represent DNA terminated and the odd ones ribbon terminated layers.

### 8.5 TEM tomography

In order to determine if specific layers can be functionalized with bis-urea modified biotin, a LbL film consisting of 5 double layers was deposited on a TEM grid. The first deposited aggregate layer (designated [ribbon/DNA]₀) was composed of biotin functionalized aggregates of the 2-D crystal like morphology as described in chapter 6. After deposition of the first layer the TEM grid was incubated with 15 nm gold-labeled streptavidin. Subsequently, the LbL process was continued until the final film thickness of 5 double layers was reached, after which 25 nm gold particles were deposited on top of the film. Low magnification TEM images showed overlapping sheets, of which many were decorated with 15 nm gold particles (Figure 8). The observation that the 15 nm gold particles do not cover the entire surface is related to the fact that also in this case the first deposited biotin functionalized aggregate layer was not fully
closed. Higher magnification images again revealed the presence of the DNA related patches (Figure 8).

![Image](image_url)

**Figure 8.** Transmission EM images of 5 double layer LbL film deposited on a TEM grid, in which the first deposited aggregate layer was functionalized with biotin and incubated with 15 nm gold labeled streptavidin. The image clearly shows many overlapping large aggregate sheets and the presence of both 15 nm biotin bound gold particles and 25 nm surface bound gold particles. Circles highlight the DNA related patches.

To determine the position of the 15 nm gold particles and thereby indirectly the biotin functionalized sheets, a tomography series was recorded. 3-D Reconstruction of the volume indeed confirmed that the 25 nm gold particles, which were deposited on top of the complete 5 double layer film, were positioned higher in the reconstructed volume than the 15 nm gold particles (Figure 9). When the z-cross-section is positioned near the substrate surface, the presence of 15 nm biotin bound gold conjugates can be clearly distinguished (Figure 9 A/C). When the z-slice is positioned at the coating surface, 25 nm gold conjugates and the DNA related patched are observed, whereas the 15 nm gold particles are absent (Figure 9B/D). As estimated from the size of a single 25 nm gold particle in the y-cross-section, the distance between the 15 nm and 25 nm particles amounted to ~30 nm (Figure 9B). Since the 15 nm gold particles are on top of the first 6 nm deposited ribbon layer, the total thickness of the 5 double-layer film is estimated to be in the order of 50 nm. The resulting single double-layer thickness of ~10 nm suggests, taking into account a thickness of 6 nm for the ribbons, that the thickness of DNA layers is ~4 nm, which amounts to ~1.5x the thickness of a single DNA chain.
Although improvement of aggregate surface coverage is needed to fully demonstrate the effect, these results do illustrate that the biotin functionality remains present in a predetermined layer during the LbL procedure and that also the layered structure is maintained as was evident from the clearly overlapping sheets combined with the spatial separation between the biotin bound and surface deposited gold particles.

**Figure 9.** Reconstructed y and z-slices of the right image in figure 8 showing: A. Lower part of the LbL coating with the z-slice positioned at the 15 nm biotin bound gold conjugates. B. Same as A, with the z-slice positioned at the surface of the coating showing two 25 nm gold particles (1 and 2) and DNA patches. Y-slice shows 25 nm gold particle 1 highlighted. From the diameter of 25 nm gold particle 1 it can be estimated that the 15 nm biotin bound and 25 nm surface bound gold conjugates are separated by approximately 30 nm in the z-direction. C. Same as A only with the x and y direction positioned at the second 25 nm gold particle 2. D. Same as B only positioned at 25 nm gold particle 2.

### 8.6 Biological analysis of the coating

In view of the potential application of these LbL DNA films as a biomaterial coating, the in vitro cell response of rat primary dermal fibroblasts (RDF) was evaluated based on cell proliferation and viability. Glass substrates were coated with 7 and 5 double layers (designated [ribbon/DNA]7 and [ribbon/DNA]5), which are DNA-terminated. RDF cells were seeded both on coated as well as non-coated substrates. At 1, 3, 7 and 10 days after cell seeding, the cell number was determined, which showed a significant increase in cell proliferation at almost every assessed time point on all coated samples with respect to the non-coated controls (Figure 10A). Also the initial cell adhesion, which was determined within the first 24 hours, showed a significant increase for the coated samples compared to the non-coated substrates.
(Figure 10B). Both 7 and 5 double-layer coatings gave similar results. The polymer based [PAH/DNA]- and [PDL/DNA]-coatings reported in chapter 4 also showed an increased cell proliferation, however, these coatings were of a mixed composition. The surface composition of the present [ribbon/DNA]-coatings consists of only DNA, which now validates the hypothesis that the observed biological effects may be due to the presence of DNA.

![Graph A](attachment:image1.png)

![Graph B](attachment:image2.png)

**Figure 10.** A. Cell proliferation of primary RDF cells on [ribbon/DNA]-coated glass plates and non-coated controls showing a significant increase in proliferation at all measured time points. B. Initial cell adhesion studies on [ribbon/DNA]-coated glass substrates and non-coated controls showing a significant increase in cell adhesion within the first 6 hours.

The morphological appearance of the RDF cells on both coated and non-coated substrates was evaluated after a 3- and 10-day culture period using scanning electron microscopy (SEM). It was observed that the coating does not significantly alter the morphological appearance of the cells compared to the non-coated controls (Figure 11). In general, all cells had a typical fibroblast appearance with several (short) cellular extensions. In addition, the coated samples showed larger cell coverage already after a 3-day culture period (Figure 11A/B), which was even more pronounced after a 10-day period (Figure 11C/D), where the coated samples are completely confluent and the non-coated controls still show uncovered regions. These data support the observed increase in cell proliferation.
To assess the RDF cell viability and cytotoxicity of the coating, a Live/Dead® assay was performed. Generally, the primary RDF cells cultured for 2 days on the multilayered DNA-coatings and non-coated controls demonstrated high numbers of live (green) cells and hardly any dead (red) cells were observed. No apparent difference in the number of dead (red) cells was observed between non-coated control samples and multilayered DNA-coatings. On the other hand, an apparent higher number of live (green) cells was observed on the multilayered DNA coating compared to non-coated control samples, which is again in line with the observed increased cell proliferation (Figure 12A/B).
Figure 12. A. Representative fluorescence images of RDF cells, cultured for 2-days on non-coated control substrates (20x magnification). B. Same as A only on [ribbon/DNA]-coated substrates.

Figure 13. β-Galactosidase determination showing the expression of the transfected plasmid DNA encoding for β-Gal. (depicted as relative light units from chemoluminescence arising from β-Gal enzyme activity) with respect to increasing DNA-surfactant lipoplex cation/anion charge ratio. Inset: enlarged section of assessed cation/anion charge ratios with respect to the negative control showing a equal or even smaller chemoluminescence.

In order to determine whether the bis-urea surfactants had gene transfer properties, which would be unfavourable, lipoplexes with DNA encoding for GFP or β-galactosidase (β-gal.) were made using cation/anion charge ratios ranging from 1.2 to 40. GFP was found not to be expressed when Hela cells (T-Rex) were incubated for 6 hours with the corresponding lipoplex, which was evident from the absence of fluorescence. β-Gal. was not expressed either for any of the assessed charge ratios and ratios higher than 4.8 scored even lower than the
negative controls, which was most likely caused by a reduction in live cells due to an increased toxicity of the surfactant at these higher concentrations (Figure 13). The positive controls did show fluorescence and a relative high β-galactosidase activity.

8.5 Conclusion

We have demonstrated that it is possible using the LbL deposition technique, to obtain a layered structure on a nanometer scale when the polymer cationic component is replaced by stable cationic surfactant bilayer aggregates. Multilayer DNA-surfactant coatings could be constructed with a linear build up. In contrast to polymer based LbL systems, a denser drop cast first layer deposition did not result in a more effective consecutive layer build-up. Only the first DNA layer showed an increase in the amount of deposited DNA, whereas the additional added ribbon layers showed a smaller consecutive amount of deposited DNA equal to that observed for a less effective first layer deposition. This indicates that the efficiency of the LbL procedure in this system is solely dependent on the ribbon coverage in each individual deposition step. The fact that the deposition efficiency of the first layer does influence the efficiency of the next DNA layer but not of the following ribbon and DNA layers, further illustrates the formation of a truly layered system without diffusion.

This conclusion was confirmed by AFM and XPS experiments, which showed that DNA is always on top for DNA terminated layers and in regions which are not covered by ribbon aggregates. Although, the surfactant aggregates did not form completely closed layers, DNA molecules did, which ensures that cells growing on a DNA terminated layer are exclusively in contact with DNA, providing a solid basis to ascribe the observed biological effects to the presence of DNA at the surface.

Furthermore, it was possible to selectively incorporate a biotin functionality into a predetermined layer within the film structure and it was demonstrated that the functionality remained present at the predetermined position during and after the LbL procedure. This system, however, can be optimized by improving the surface coverage of the aggregate layers, since the DNA layers are not the limiting factor. This might be achieved by growing even larger sheets (see chapter 5), which are then used in the LbL deposition procedure.

Finally, it was shown that these DNA-based coatings are cyto-compatible and show a significant increase in cell proliferation, which was now confirmed to be due to the presence of DNA. Furthermore, the bis-urea surfactant 1 did not induce transfection providing a solid basis for the further development of DNA-based biomaterial coatings.
8.6 Experimental.

**General materials and equipment.** The Dutch Spinoza Junior Researchers Institute supplied large quantities of bis-urea surfactant 1, synthesized via the CDI route. Polyamionic DNA (~300 bp/molecule; sodium salt) was kindly provided by Nichiro Corporation (Yokosuka-shi, Kanagawa prefecture, Japan). Potential protein impurities in the DNA were checked using the BCA protein assay (Pierce, Rockford, Illinois, USA) and measured to be below 0.20% w/w. All solvents were purchased from Acros Chemica or Sigma-Aldrich and of p.a. quality. Aqueous solutions were always prepared using Ultra pure Milli-Q water (Barnstead EASYpure LF system; R >17.7 MΩ-cm). A 1 mg/mL aqueous solution of DNA was prepared by dissolving the supplied freeze dried DNA in ultra pure Milli-Q water. The 1 and 5 mg/mL ribbon solutions were prepared by suspending freeze dried surfactant 1 in ultra pure milli-Q water and subsequent heating to 90 °C resulting in a clear solution. Cooling to room temperature yielded the turbid ribbon solution. Glass and quartz substrates were cleaned and negatively charged prior to LbL film deposition, by immersing in NaOH/Ethanol solution for 30 min. and subsequent rinsing with Milli-Q water for 10 min. Silicon wafers and TEM grids were, when indicated, additionally treated with a 75 Watt oxygen plasma for 5 min. after cleaning with NaOH/ethanol solution as described above. Eagle minimum essential medium (EMEM) was enriched with foetal calf serum (FTS), penicillin and glutamax to obtain EMEM+ and only enriched with glutamax for EMEM-. Reporter Lysis Mix (RLM) is composed of 25mM Bicine, 0.05% Tween 20 and 05% Tween 80 in Milli-Q water. Galacornix is composed of 6.25 mL demiwater, 1.6 mL 0.5M phosphate buffer (pH: 8.2), 40 μL 1.0M MgCl2 in water and 80 μL Galacton. EMEM (BioWhitaker TM) was purchased from Cambrex Bio Science, Glutamax (100x) from Invitrogen, Pen Strept (500x) from Roche Diagnostics Netherlands, Foetal Bovine Serum Gold from PAA via New Brunschwig, Galacton Plus from Applied Biosystems and Accelerator II from Applied Biosystems. pDNA was synthesized using own maxiprep equipment.

UV-vis spectra were recorded on a Shimadzu Multispec-1501 equipped with a diode-array detector. XPS measurements were performed using a non-monochromized VG Escalab 200 spectrometer equipped with an aluminum anode (AL KR; 1486.6 eV) operating at 510 VA with a background pressure of 2 x 10⁻⁹ mbar. Spectra were acquired at 0° and 60° with respect to the surface normal. AFM images were recorded on a Veeco Multimode equipped with a Nanoscope IV control unit and Nanosensors tips with resonance frequencies of 300 kHz operating in the tapping mode regime. Microfabricated silicon cantilevers were used with a spring constant of 30 Nm⁻¹. The instrument was equipped with the Extender™ Electronics Module to provide simultaneously height, amplitude and phase cartography. Transmission microscopy measurements were performed on a FEI Tecnai 20, type Sphera TEM operating with a 200 kV LaB₆ filament. Images were recorded with a bottom mounted 1k x 1k Gatan CCD camera. TEM tomography measurements were performed on a FEI Titan TEM equipped with a field emission gun (FEG) operating at 300 kV. Images were recorded using a Gatan GIF energy filter and a 2k x 2k Gatan CCD camera. The tomography reconstruction was performed using the Inspect3D (FEI company) software program version 2.1. 15 Nm gold-labeled streptavidin and 25 nm gold particles, incubation block buffer and 200 mesh carbon coated copper grids were purchased from Aurion.

**LbL deposition and chemical analysis**

**Coating deposition.** The LbL coatings were constructed using alternate absorption from an aqueous 1 mg/ml ribbon solution and an aqueous 1 mg/ml DNA solution, with immersion times of 7 min. followed by a wash step of 3 min. rinsing using Milli-Q water. Two methods of first layer deposition were used: (1) 30 min. immersion in a
concentrated 5 mg/ml ribbon solution followed by a 3 min. wash step, (2) a drop cast layer using a 1 mg/ml ribbon solution without a subsequent wash step and drying using a stream of nitrogen. LbL films used for the presented analysis were prepared using only method 1 as a first layer deposition unless indicated otherwise.

**UV-Vis.** For the UV measurements, 10 double-layers (ribbon-DNA) were deposited on a NaOH/ethanol cleaned quartz substrate and measured after each successive layer using a Shimadzu multispec UV-Vis spectrophotometer (Shimadzu Germany, Duisburg, Germany), equipped with a diode-array detector. The DNA spectra were corrected for scattering using the measured spectra of the ribbon terminated layers and the incremental increase of the 260 nm nucleic base chromophore was plotted versus the number of deposited double-layers.

**Atomic Force Microscopy (AFM).** Different LbL films on glass were prepared for AFM measurements, composed of 1 to 12 single layers. All the AFM images were recorded with a Nanoscope IV microscope from Veeco Inc., operating in intermittent contact (in air at room temperature). Images of different areas of each sample were recorded. The Nanoscope image processing software was used for image analysis. Height images were used to determine the general topology of the sample, whereas amplitude images were used to determine which structure was on top.

**X-ray photoelectron spectroscopy (XPS).** Different LbL films on glass were prepared for the XPS using method 1 as first layer deposition, composed of 1 to 8 single layers (odd layers are ribbon-, even layers are DNA terminated). XPS spectra were measured at 0° and 60° with respect to the surface normal. The phosphorus/nitrogen ratios were calculated using the integrated intensities and plotted versus the number of single layers both for 0° and 60°. To improve on the layer deposition and XPS spectra, silicon wavers were cleaned with a NaOH/Ethanol solution and treated with a 75 Watt oxygen plasma as described in the general materials section. Subsequently a first ribbon layer was deposited from a 5 mg/ml ribbon suspension onto the silicon substrates after which excess fluid was removed with a stream of nitrogen. Next 2-8 additional layers were deposited using the standard LbL procedure described above, only the wash step was reduce by 1 min. immersion in Milli-Q water followed by quick drying using a stream of nitrogen. All eight sequential layers were measured using XPS both at 0° and 60° and the phosphorus/nitrogen ratios were calculated using the integrated intensities and plotted versus the number of single layers.

**Transmission electron microscopy**

**Deposition efficiency.** A specially prepared TEM grid, which was etched from a silicon waver, was cleaned with NaOH/ethanol solution and treated with a 75 Watt oxygen plasma for 5 min. A ribbon layer was deposited according to the dropcast method 2 and imaged on the Sphera TEM. After imaging the grid was washed for 3 minutes in milli-Q water, dried using a stream of nitrogen and image again using the Sphera TEM. A second ribbon sample was prepared using method 2, without washing and immersed in a 1 mg/mL DNA solution for 7 min. followed by a 3 min. wash step in milli-Q water. The sample was subsequently dried and imaged using the Sphera TEM.

**TEM tomography.** A 5 double-layer LbL sample was prepared on a conventional carbon coated 200 mesh copper grid. To this end droplets of alternating a matured\(^1\) 0.5 mg/ml ribbon solution or a 1 mg/ml DNA solution were placed on a TEM grid for 7 min. After each ribbon or DNA solution the grid was washed by placing a droplet of milli-Q water for 3 min. on top. After each time period (either the deposition or the wash step), the grid was partially blotted from the side to remove only the excess fluid using a filter paper. Layer number 1 (first ribbon layer) was deposited from a 0.25 mg/ml biotin functionalized 2D crystal-like aggregate solution and subsequently incubated using 15 nm gold-labeled streptavidin as described in chapter 6. The 5 double-layer coating was topped with 25 nm gold particles to enable feature tracking in the final reconstruction procedure. After the last gold deposition step, the

\(^1\) To increase the amount of broad sheets a four months old solution was used.
samples were blotted completely and subsequently dried in high vacuum. TEM images both for standard in transmission as well as in tomography were recorded using low-dose conditions at -170 °C. The region of interest was selected and positioned optimized for a maximal tilt angle to both sides. The tilt series was recorded on a Titan microscope operating at 300 kV using a GIF energy filter together with the full range of the 2k x 2k CCD camera. The Xplore3D software was used to acquire the tilt series.

**Biological Assays**

**Cell culture, seeding, proliferation and morphology.**
The biological experiments were performed using 5 and 7 double-layer coatings deposited on glass. Untreated glass substrates were used as control. Both 7 and 5 double layered coatings gave similar results. The applied procedures have been described previously and have not been unaltered to ensure proper comparison with previously described work.

**Cell culture and seeding.** Rat dermal fibroblasts (RDF’s) were obtained from the ventral skin of male Wistar rats, using a standard procedure as described previously, after which cells were cryo-preserved. Before the initiation of an experiment, cells were thawed and cultured in culture medium (α-MEM (Gibco), supplemented with 10% (v/v) fetal calf serum and gentamycin (50 μg/mL)). All assays were performed with cells of the fourth or fifth culture passage. Prior to the assays, cells were detached using trypsin/EDTA (0.25% w/v trypsin / 0.02% EDTA) and concentrated by centrifugation at 1500 rpm for 5 min. Subsequently, cells were resuspended in culture medium and counted using a Coulter counter (Beckman Coulter Inc., Fullerton, CA, US). For all in vitro assays, cells were seeded at 1 x 10⁴ cells/substrate into 24-wells plates (Greiner Bio-One BV, Alphen aan de Rijn, the Netherlands).

**Cell morphology.** RDF’s were cultured on the experimental substrates and coated controls using culture medium. After 3 and 10 days, the substrates were washed twice with PBS. Subsequently, cell fixation was carried out for 15 min in 2% glutaraldehyde in 0.1 M sodium cacodylate buffered solution. Then, the substrates were rinsed twice with cacodylate buffered solution and dehydrated using a graded series of ethanol. Finally, the substrates were dried using tetramethylsilane. The samples were sputter coated with gold and examined using a JEOL 6310 SEM.

**Live/Dead® assay.** A commercially available kit (Live/Dead® assay; Molecular Probes, Eugene, OR, US) was used to evaluate the viability of the primary rat dermal fibroblasts (RDF’s) on the experimental substrates and coated controls, according to the instructions of the manufacturer. Briefly, after 2 days of cell culture, cells were washed twice with PBS (Phosphate Buffered Saline; pH 7.4). Subsequently, cells were incubated in appropriate amounts of fluorescent dye for 45 min at 37 °C. Then, cells were rinsed twice with PBS and visualized using a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany), equipped with a digital camera.

**Transfection assay.** The transfection assay was performed using a standard protocol. HeLa cell-line (T-rex) were seeded into 6-wells plates and cultured for 1 day until ~20% confluent. Transfection was performed in EMEM- to inhibit mitoses. After addition of the transfection-mix, the cells were incubated for 6 hrs. Subsequently, the transfection-mix and EMEM- medium were replaced by EMEM+ and the cells were left for 45 hrs. to multiply. Following the transfection procedure, the cells were then analyzed using fluorescence microscopy to determine expression of GFP and analyzed for the expression of the enzyme β-galactosidase. To each well 100 μL transfection-mix was added composed of 1 μL plasmide DNA (pDNA)(1 μg/μL) lipoplex solution in 100 μL EMEM+. The pDNA solution was composed of 10% EGFP (2.5 μL), 10% CMV β-Gal (2.5 μL) and 80% Blue Script (20.0 μL) in 2500 μL.
EMEN-. The Blue Script was added to prevent overexpression. Lipoplex solutions were prepared by mixing 200 μL p(DNA) solution with 200 μL EMEM- with addition of the calculated amount of surfactant 1 to obtain final cation/anion ratios of 1.2, 2.4, 3.6, 4.8, 10, 15, 20, 25, 30 and 40. Di-oleyl-phosphatidylethanolamine/ Dimethyl-dioctadecylammonium Bromide (DOPE/DDAB) was used as the positive control surfactant with a cation/anion charge ratio of 1.2. DOPE stimulates cell membrane permeability and DDAB is used for condensing DNA. The lipoplex solution without addition of surfactant was used as the negative control. The well plates incubated with a 1.2-3.6 cation/anion ratio transfection-mix were completely confluent after 45 hrs cell growth. Ratios of 4.8 and 10 cat/an showed significantly less cell coverage (~50%), whereas charge ratios higher than 10 showed almost no deposited cells (~10%). The positive control was 50% confluent and the negative control was fully covered.

For the β-gal determination, 200 μL of RLM mix was added to each of the wells to puncture the cell wall in order to release the galactosidase enzyme. Subsequently, the cells were thoroughly suspended in the well solutions and 20 μL was transferred into a separate tube. To each tube 200 μL gallactonmixin was added and left for 30 min. after which Eccelerator™ was added to enhance the chemo-luminescence. After mixing the tubes were measured in a luminometer and values were presented in relative light units. The chemo-luminescence originates from the hydrolysis of galactosidase by galacton yielding a chemo-luminescent group. The protein GFP and the enzyme galactosidase are found naturally in HeLa cells. From all results obtained, only the positive control showed expression of GFP and β-gal.

### 8.7 References

DNA-Surfactant Complexes as a Biomaterial Coating

Summary

All known life on earth stores its genetic information in double stranded DNA. Such information is conferred through sequences of nucleotide pairs, derived from the four ubiquitous purine or pyrimidine bases. This unique property of information storage has found applications in many fields of research from sensor arrays to the construction of well-defined molecular aggregates. Until now, the use of DNA as a coating for biomaterials applications has been virtually unexplored. We became interested in DNA for many of the reasons already mentioned, its degradability profile, but also for the non-immunogenic properties of vertebrate DNA. Since DNA has a high solubility in aqueous media and is easily degraded by nucleases in vivo, it is highly desirable to develop a coating method that both immobilizes DNA at the surface of a biomaterial implant and simultaneously reduces the susceptibility to enzymatic degradation without affecting the beneficial non-immunogenic properties of DNA.

The main objective of the research described in this thesis was to find a suitable technique to produce DNA based biomaterial coatings and analyze the coating structure as well as the biological effect. The possibility to incorporate other molecules into the coating structure for future drug release or cell signaling and/or differentiation purposes has also been investigated.

Spin-coated complexes of DNA and cationic surfactant molecules did not yield the desired coating, nor did the Langmuir Schaefer deposition of polymerizable DNA-surfactant Langmuir monolayers (chapter 3). It was therefore decided to investigate the Layer-by-Layer (LbL) assembly technique, which uses the repeated sequential deposition of negatively and positively charged components onto a substrate of any shape via a dipping procedure. Using the LbL technique, coatings were constructed based on DNA as the anionic and PDL or PAH as the cationic polymer components. Chemical analysis of the coating showed that the structure was enriched with DNA at the surface of the film. However, distinct layers on a nanometer scale were not observed. Nevertheless, biological assays showed that these coatings were cyto- and histocompatible and induced an increased effect on cell proliferation (RDF cells), calcium deposition (after SBF: immersion) and cell differentiation (osteoblast-like cells on SBF-treated coatings). Furthermore, a decrease in the pro-inflammatory cytokine TNF-α was observed for the coated samples, providing a first indication of the possible non-immunogenic effect of the DNA-based coating. In addition, the coating could be enriched with the osteoinductive factor BMP-2, which was released again during cell culture experiments (chapter 4).

Although these experiments did show that the LbL coatings composed of DNA and cationic polymers are promising candidates for an application as a biomaterial coating, the observed biological effects can not be ascribed solely to the presence of DNA in the film due to the mixed composition of the top layer. In addition, a burst release up to 75% was observed when the coating was enriched with BMP-2 further illustrating the diffuse and mixed character of the polymer DNA films. The origin of the mixed film composition lies in the high rate of diffusion of the polymer chains during the LbL procedure (chapter 2). When the diffusion is limited theoretically, a truly layered structure on a nanometer scale should be obtained. This layered structure would ensure that, when DNA is deposited as the final layer, only DNA is present at the surface in contact with the cells. In addition, it would allow additives to be incorporated into a predetermined layer, opening up possibilities for timed drug release upon degradation of the coating. Inspired by the lamellar phase of DNA-surfactant complexes a new cationic bis-ureido based surfactant was prepared which formed bilayer structures in water. It was speculated that replacement of the cationic polymer component with these cationic bilayer aggregates would result in a truly layered coating structure on a nanometer scale. The tight, almost crystalline packing of the surfactant molecules within the bilayer structure...
caused their assemblies to behave more like crystals than like surfactant aggregates. The size and shape of the aggregates could therefore be changed from a ribbon-like morphology with high aspect ratio to a faceted 2-D crystal-like objects with a low aspect ratio, by changing the supersaturation of the solution in which they were formed (chapter 5).

DNA might be used for the incorporation of other functional molecules within the LbL structure via groove binding and intercalation. However, intercalating molecules inherently are often carcinogenic because they insert between the aromatic DNA basepairs. In addition, groove binding poses restrictions on the size of the molecules that need to bind to the helical groove and the binding is often not strong. The bis-ureido unit of the bilayer aggregates can therefore supply an additional possibility to anchor functionality into the LbL coatings. It was shown that coupling of a matching bis-ureido functionality to biotin resulted in incorporation of the biotin functionality both in the ribbon as well as in the 2-D crystal-like morphology of the bilayer aggregates. The selectivity of the molecular recognition was demonstrated by coupling a matching or non-matching bis-urea functionality to a dye molecule, in which the matching combination was more uniformly bound into the bilayer aggregates (chapter 6).

The interaction between the bis-urea surfactants and DNA was studied in a Langmuir-monolayer model system. The DNA-surfactant complex was first formed by injecting the DNA underneath a preformed monolayer of the surfactant, or by spreading the cationic surfactants on a DNA containing subphase. It was demonstrated that only in the former case a DNA-surfactant monolayer was formed in which the DNA molecules are bound underneath a closed cationic surfactant monolayer surface. It was also shown that most DNA strands do not bind completely to the monolayer surface, but have a part of the strand extending into the sub-phase (chapter 7). In the latter case, it was speculated that spreading of the cationic surfactants on a DNA subphase results in the formation of DNA-surfactant complexes that occupy the surface, rather than the formation of a closed monolayer of surfactant molecules.

Because the DNA chains do attach to a preformed monolayer surface without disrupting the molecular organization, it was anticipated that the LbL deposition should work using the bilayer aggregates, which have the same molecular packing as the monolayer surface. Indeed the LbL procedure using DNA and bis-ureido surfactant bilayer aggregates resulted in the formation of a layered coating on a nanometer scale. Although the aggregates do not form completely closed layers, it was however demonstrated that DNA terminated layers were completely covered with DNA. In addition it was demonstrated that the bis-urea modified biotin molecules could also be incorporated within a predetermined layer, and remained at this position during the LbL procedure while retaining their biological functionality. Initial biological assays on these coatings again showed an increased cell proliferation. Since it was shown that DNA was the only component present at the surface of these films this effect, which was also observed for the polymer DNA-based films, can now ascribed to the presence of DNA (Chapter 8).

Since drug delivery relies on diffusion and appropriately sized reservoirs, diffuse polymer based coatings like the [PAH/DNA] and [PDL/DNA] are best suited for this purpose. Surface signaling and timed release, on the other hand, require control over the diffusion and a layered structure as obtained with the bis-urea surfactant system. In addition, the biological effect of DNA can be more reliably studied using such a truly layered structure, in which DNA is exclusively situated in the outer most layer in contact with the cells. Since growth factors are quite often extremely expensive, the possibility of anchoring these functionalities within the coating structure without losing substantial amounts during the subsequent build-up and washing procedures of the following layers, introduces also an additional benefit. As described in chapter 2, the examples of polymer based Layer-by-Layer films intended for biomaterial applications are numerous; but to date no examples existed in which a truly layered structure on a nanometer scale was constructed. The work described in this thesis tries to fill this gap and provides the first steps in the development of such a film based on complexes of DNA and self-assembling cationic surfactants.
List of Publications


Curriculum Vitea

Matthijn Vos was born on May 25, 1977 in Nijmegen, the Netherlands. After completing the higher general secondary education (havo) at the Nederrijn College in Arnhem he started the teachers education (NLO) in chemistry at the Hogeschool Arnhem and Nijmegen (HAN) in 1994, which included a two year chemistry study at the Hoger Laboratorium Onderwijs (HLO) in Arnhem. He obtained his Bachelor degree in 1999. During this period he performed two external traineeships: as full-time chemistry teacher for the duration of 4 months at the ROC De Leijgraaf, department of Process- and Laboratory Engineering situated in Oss and as laboratory technician at NV Organon, Department of Analytical Control, section Dispensing Services & Control in Oss for the duration of 3 months. Following the traineeships he worked as a part-time teacher at the ROC De Leijgraaf for the duration of 6 months and as a part-time laboratory technician at NV Organon for the duration of 3 months. From September 1999 to August 2002 he studied chemistry at the Radboud University Nijmegen and obtained his Master degree in Bio-Organic Chemistry. In October 2002 the author started his Ph.D research at the Department of Biomedical Engineering at the Eindhoven University of Technology. In the Laboratory of Macromolecular and Organic Chemistry (SMO), under the supervision of prof.dr. R.J.M. Nolte and dr. N.A.J.M. Sommerdijk he investigated the development of DNA-based biomaterial coatings. This research was a collaboration with the department of Periodontology & Biomaterials of the Radboud University Nijmegen under the supervision of prof.dr. J.A. Jansen.

The most important results are described in this thesis. In March 2007 the author will start a post-doc research specialised in electron microscopy at the Dutch Cancer Institute in Amsterdam under the supervision of prof.dr. P.J. Peters, section Tumor Biology.
Dankwoord

Tot slot dan het meest gelezen gedeelte van het proefschrift en daarmee misschien wel het moeilijkste om te schrijven. Alhoewel het DNA-coatings project vooraf werd gezien als gedurfd en misschien ook wel riskant is het uiteindelijk toch een groot succes geworden, mede dankzij de hulp en inzet van vele anderen. Ook mijn persoonlijke ontwikkeling, met name op het gebied van de electronen microscopy, dank ik aan de betrokkenheid en inzet van veel collega’s. Mijn dank hiervoor is groot!


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Matthijn