Glycerol Based Polymers for Nonfouling and Switchable Coatings

Dissertation zur Erlangung des akademischen Grades des
Doktors der Naturwissenschaften (Dr. rer. nat.)

eingereicht im Fachbereich Biologie, Chemie, Pharmazie
der Freien Universität Berlin

vorgelegt von

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2014

Diese Arbeit wurde unter der Anleitung von Prof. Dr. Rainer Haag im Zeitraum von Dezember 2010 bis Dezember 2014 am Institut für Chemie und Biochemie der Freien Universität Berlin angefertigt.

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Disputation am 19.12.2014

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1 Introduction

1.1 Biofouling

Biofouling is defined as the unwanted settlement of biological material on solid surfaces with potentially detrimental impact on the function of the material.[1] Despite major scientific progress, biofouling remains an unresolved problem in a variety of applications including medical implants,[2-4] biosensors,[5,6] filtration membranes,[7,8] food packaging[9,10] and ship hulls.[11,12] Although biofouling on implants and ship hulls seem to be unrelated at first sight, both processes share a similar mode of action. Where the initial step upon contact with biofluids for biomedical devices is the adsorption of proteins, marine organism such as barnacles and blue mussels adhere with protein adhesives.[13,14] In both biomedical and marine applications fouling has severe consequences for the performance of the respective material. In the case of medical implants, the adsorbed proteins serve as a provisional matrix that initiate blood coagulation or a foreign body reaction.[15] Adhesion of marine organisms, on the other hand, considerably increases the hydrodynamic drag of the ship vessel and leads to increased fuel consumption.[16] Hence, prevention of protein adsorption is generally a sufficient but not necessary prerequisite for reducing biofouling. A typical approach for rendering substrates protein resistant is surface modification with polymeric materials (Figure 1).[17] Whereas *in vitro* diagnostic applications such as biosensors or protein microarrays almost exclusively use non fouling approaches that are based on minimizing adhesion to proteins, coatings with an active biocidal agent are applied in marine environments to prevent the settlement of fouling organisms.[18] Due to the restrictive legislation for biocides and therewith associated increased cost for biocide development, fouling release coatings (FRCs) that minimize the adhesion of fouling organism are gaining a share of the market.[18]
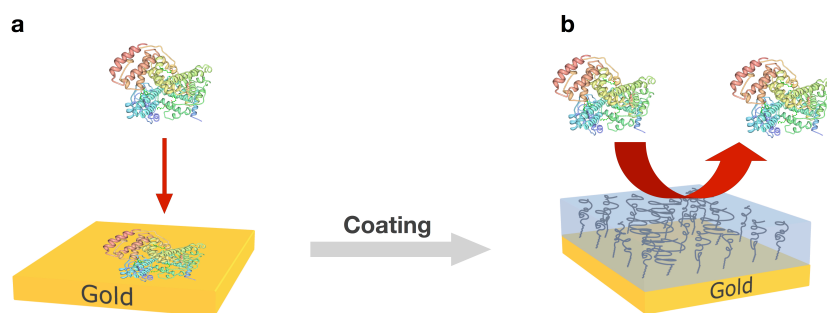
Figure 1. (a) Nonspecific protein adsorption on uncoated surface and (b) protein resistant surface after coating with a hydrophilic polymeric layer.

Since this work describes the development and application of protein resistant coatings, the following sections describe the underlying mechanism and design strategies for protein resistant behavior. High surface energies and strong surface hydration of the surface seem to be necessary prerequisites for nonfouling behavior, but not a sufficient one as indicated by a strong protein adsorption on charged hydrogels.[17] The correlation between hydrophilicity and protein resistance is reflected in the concept of Berg's limit which classifies surfaces according to their water contact angle into hydrophobic and hydrophilic surfaces with a transition at approximately 65°.[19,20] Above Berg's limit, the dominating attractive hydrophobic forces render the surface protein adsorptive, while below it, the hydration repulsion considerably decreases the protein adhesion strength.[21] On a molecular level the adsorptive behavior of hydrophobic surfaces can be described by an energetically favorable expulsion of water molecules. In contrast, the displacement of water molecules on hydrophilic surfaces is energetically unfavorable due to the high surface energies close to the value of water.[22] The protein repellency of highly hydrophilic polymers is also ascribed to a steric repulsion caused by the high mobility of the tethered polymer chains. When a protein approaches the surface, the amount of conformational states for the tethered polymer chains becomes less which restricts the chain mobility and is therefore entropically unfavorable.

In order to set up a structure-activity relationship, Whitesides and coworkers systematically tested 50 functional groups for their ability to resist the adsorption of proteins.[23] Therefore, they immobilized a broad range of amine bearing low molecular weight compounds on self assembled monolayers (SAM) that had a terminal anhydride group and they tested the adsorption of fibrinogen and lysozyme with surface plasmon resonance (SPR) spectroscopy. The authors concluded that protein resistant surfaces should (i) be hydrophilic, (ii) contain groups that are hydrogen bond acceptors, (iii) not contain groups that are hydrogen-bond donors, and (iv) be overall electrically neutral. Although these rules are widely accepted, several compounds that resist the adsorption of proteins do not match these criteria. For instance, hydroxyl terminated poly(ethylene glycol) (PEG),[24] dextran,[25] and polyglycerol (PG)[26] bear hydroxyl groups that can act as hydrogen bond donors. These exceptions show that the underlying principles of protein repellency are still poorly understood.[*]

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[*] This paragraph is a revised version of a paragraph from the author's Master's thesis.

As the coatings described within this work are based on various PG derivatives, the following section gives an overview on the current state of PG coatings.

1.1.1 Bioinert glycerol based polymeric coatings

Glycerol based polymers belonging to the family of aliphatic polyethers are commonly prepared with linear and branched structures via anionic ring-opening polymerization (Figure 2).[27,28] The synthetic procedure for dendritic polyglycerol (dPG) makes use of the latent hydroxyl group in glycidol to obtain branched structures, whereas the synthesis of linear polyglycerol (lPG) is based on the protection of this very group. In contrast to PEG, which is also an aliphatic polyether, both linear and branched polyglycerols exhibit a high number of hydroxyl groups, which can be used for cross-linking, labeling, or incorporating of targeting and grafting moieties.[29] Additionally, these groups can be modified before or after polymerization to adjust the physical and chemical properties of the polymer.[30] Due to the structural similarity of their backbones, PGs have many features in common with PEG, which is considered as the gold standard for rendering surfaces protein resistant. However, several differences have been observed for PG such as a higher resistance to heat and oxidation[17] as well as reduced platelet and complement activation.[31]
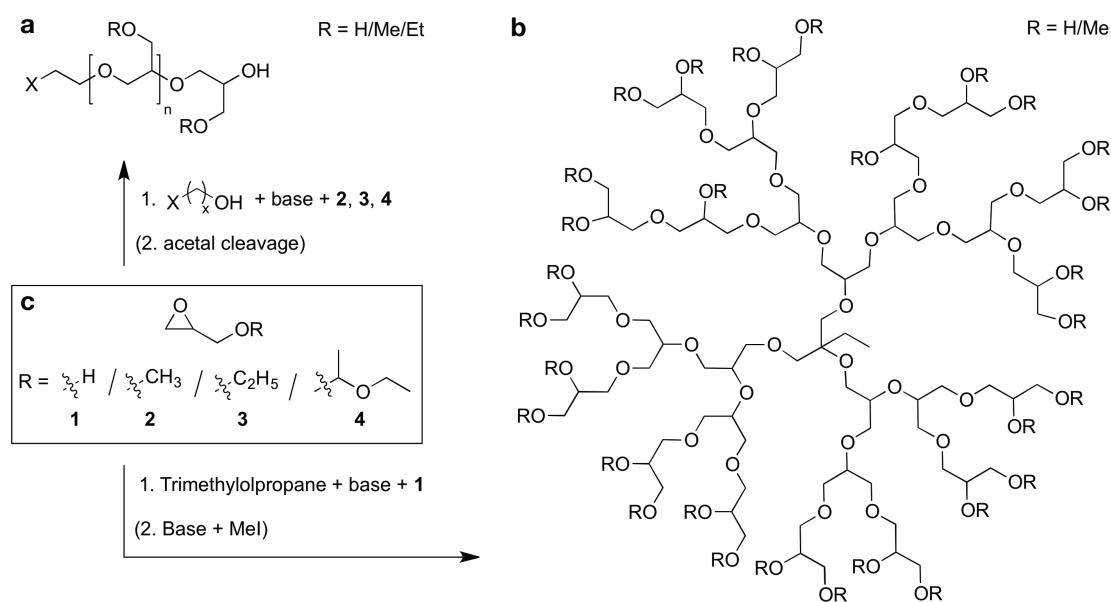
Figure 2. (a) Linear polyglycerol with hydroxyl, methoxy, and ethoxy side groups, respectively. (b) Dendritic polyglycerol with hydroxyl and methoxy side groups, respectively. (c) Glycidol (R = H), glycidyl methyl ether (GME) (R = CH$_3$), glycidyl ethyl ether (EGE) (R = C$_2$H$_5$) and acetal protected glycidol, namely, ethoxy ethyl glycidyl ether (EEGE) (R = CH(CH$_3$)OCH$_2$CH$_3$).

The first approach using PG to render surfaces resistant against the adsorption of proteins was based on dPG, which generally exhibits a branching degree of about 60% (Figure 3).[32] Haag and coworkers synthesized dithiolane functionalized dPGs and immobilized them on gold surfaces.[26] dPG monolayers showed an excellent resistance against the adsorption of fibrinogen. To test the validity of the Whitesides rule that absence of hydrogen bond donors should improve protein repellency, 88% of the hydroxyl groups of dPG were transformed to methoxy groups. However, surfaces modified with methoxylated dPG (dPG(OMe)) did not show an improved protein resistance compared to dPG. Surprisingly, dPG(OMe) exhibits very poor water solubility which suggests that an interfacial water layer is not necessary for rendering surfaces protein resistant. Another approach to render gold surfaces protein resistant using dPG was presented by Kizhakkedathu and coworkers.[33] Monothiol terminated dPGs having molecular weights of 1.5 and 4 kDa were synthesized by anionic ring-opening polymerization using 2,2′-dihydroxyethane disulfide as the initiator and subsequent reduction of the disulfide. The authors could show that the high molecular weight dPG was more resistant to bovine serum albumin (BSA) adsorption than PEG with similar molecular weight and low molecular weight dPG. Subsequently, Haag and coworkers tested lPG and methoxylated lPG (lPG(OMe)) for their protein resistant properties.[27] Therefore, lPG and lPG(OMe) with a terminal thiol group that enabled immobilization on gold were prepared and compared to PEG modified surfaces with respect to protein

and serum adsorption as well as cell adhesion. Whereas in the case of single protein adsorption, PEG and lPG coated surfaces showed excellent protein resistance, lPG performed significantly better when the resistance against human serum was tested. To further evaluate PG coatings with respect to their nonfouling behavior and to demonstrate the applicability of PG coatings to other materials, Haag and coworkers synthesized triethoxysilyl modified PGs and tested correspondingly coated glass for their ability to resist protein, cell, and bacteria adhesion.[34] Both PEG and PG monolayers on glass showed a considerable reduction of bovine serum albumin (BSA) and fibrinogen adsorption (more than 90% after 24 h). Furthermore, they showed only a negligible amount of adhesion of mouse fibroblasts after 1 week incubation and excellent resistance against gram-positive and -negative bacteria strains. In order to establish a structure-activity relationship for glycerol based structures, Haag and coworkers synthesized linear and branched glycerol based compounds with a defined number of repeating units (Figure 3). They then immobilized these compounds on gold and tested them for their ability to resist the adsorption of various proteins.[35] In the case of linear glycerol oligomers, three repeating units were necessary to achieve a level of fibrinogen adsorption 10% below the adsorbed amount on hydrophobic 1-hexadecanethiol (HDT) coated substrates, and in the case of dendrons the second generation was necessary to achieve the same level.
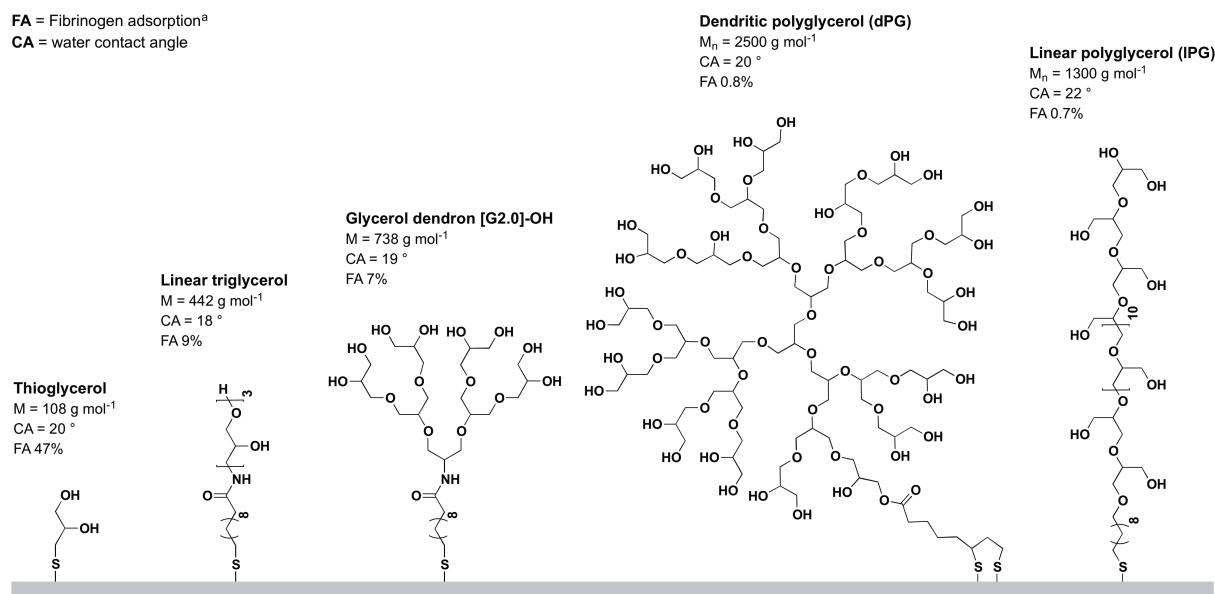
Figure 3. Protein resistant behavior of various glycerol based structures that were tested by the Haag group for their ability to resist the adsorption of fibrinogen. All compounds were immobilized on gold slides and tested for the adsorption of fibrinogen via surface plasmon resonance (SPR) spectroscopy with a fibrinogen concentration of 1 mg mL^{-1}. FA = amount of fibrinogen adsorption relative to the amount of adsorbed fibrinogen on 1-hexadecanethiol (HDT) modified slides.

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As this work describes the development and testing of nonfouling coatings in biomedical and marine applications, the following two chapters will further discuss nonfouling approaches for *in vitro* diagnostics, primary packaging, handling of biopharmaceuticals and marine applications.

1.1.2 Fouling in biomedical applications

The following two sections are revised versions of two chapters written by the author and published in the review article: Qiang Wei, Tobias Becherer, Stefano Angioletti-Uberti, Joachim Dzubiella, Christian Wischke, Axel T Neffe, Andreas Lendlein, Matthias Ballauff, and Rainer Haag, *Protein Interactions with Polymer Coatings and Biomaterials, Angew. Chem. Int. Ed.* **2014**, *53, 8004-8031.*

In vitro diagnostics

Coatings used for solid-phase immunological assays mainly have to fulfill two criteria. They must reduce the nonspecific adsorption from protein solutions or complex media like blood plasma as well as allow for the immobilization of a protein or peptide ligand. The simplest approach for the immobilization of proteins is physisorption based on hydrophobic interactions with the PS substrate, which is mostly used for microtiter plate assays. To account for nonspecific adsorption, the remaining binding sites are subsequently blocked by applying detergents or protein based blockers. In the case of protein microarrays, physisorption to nitrocellulose cast glass surfaces is the generally applied method, because the porous structure of nitrocellulose enables high ligand density and stabilization of the ligand.[36] Similar to microtiter plate assays, the remaining binding sites are blocked by suitable blocking buffers. The simplicity of these methods, however, can cause increased nonspecific adsorption and denaturation of the immobilized ligand,[37,38] which correspondingly leads to higher background noise and decreased signal intensity. Additionally, the lack of regenerability of the ligand-coated surface after each measurement cycle presents a problem when determining binding or rate constants by means of time-resolved detection methods like surface plasmon resonance (SPR) spectroscopy or quartz crystal microbalance (QCM). A protein-repellent polymeric matrix that offers suitable reactive groups for ligand immobilization addresses all of the above-mentioned problems.

Since glass, a commonly applied substrate for protein microarrays and oxidic materials, aplied in optical waveguide lightmode spectroscopy (OWLS) and in silicon microring resonators,[39] share the

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same functional silanol groups on the surface, these surfaces can be functionalized by means of silane chemistry. The simplest way to modify SiO_2 surfaces is to apply standard bifunctional silane coupling agents like 3-aminopropyltriethoxysilane (APTS), 3-isocyanatopropyltriethoxysilane (ICPTES), or (3-glycidyloxypropyl)triethoxysilane (GOPTES). These agents can be used for direct modification with a specific biomolecule or as adhesion promoters for the further functionalization with an inert matrix. Apart from lacking an inert matrix, many of the primary amines needed in the first approach may not be accessible due to steric hindrance. Pathak et al. addressed this problem by using 1,1'-carbonyldiimidazole on glass and silicon surfaces as an adhesion promoter for subsequent modification with polypropylene imine (PPI) dendrimers of generation 1-5.[40] The authors showed that the accessibility of the amines significantly increased with higher generations. In order to address the problem of nonspecific adsorption, Hucknall et al. used ATRP to graft up to 100 nm thick poly[(oligo(ethylene glycol))-g-methacrylate] brushes onto glass surfaces and applied them in protein microarrays with a fluorescence readout.[41] By comparing these coatings with commercially available nitrocellulose membranes, the authors demonstrated that the reduction of the background signal permitted limits of detection (LODs) that were a hundred-fold lower. When the polymer was grafted onto a gold surface, the nonspecific adsorption from undiluted bovine serum was below the detection limit of a commercial SPR device. Additionally, the authors showed that ligand immobilization could be achieved without a covalent coupling, just by physical entanglement of the grafted polymer chains. A promising alternative to hydrolytically unstable siloxane bonds is the photochemical grafting of terminal alkenes to silicon and silica surfaces.[42,43] This approach is also applicable to carbon-based materials as well as other semiconductor surfaces like germanium, gallium, and nitride.

Three-dimensional polymeric layers generated by "grafting from" approaches, as applied by Hucknall et al., are also of particular interest in evanescent field based biosensors as the height can be easily adjusted to fit the decay length of the evanescent field. However, the coating density has to be kept low enough to allow penetration of the ligand and analyte molecules. In time-resolved methods, like SPR or QCM, the nonspecific adsorption is generally compensated by running a reference channel in contact with the same analyte solution and by subsequently subtracting the reference signal from the measured signal. However, the accuracy of the data is considerably higher, when nonspecific binding is minimized.[44] For commercial SPR chips, this is generally achieved by applying a coating of carboxymethyl dextran (CM-dextran), a semisynthetic polysaccharide, in which the carboxylic groups can be used for ligand immobilization. Unfortunately, the reference compensation and the protein-resistant coating do not take the nonspecific adsorption that is introduced by the ligand into account. An alternative referencing approach was suggested by Špringer et al.[45] Instead of using an unmodified

matrix-coated channel as reference, the detection channel with the immobilized ligand was exposed to an analyte, whose binding sites were blocked with the same ligand. A substrate-independent approach for the generation of functional surfaces was presented by Rodriguez-Emmenegger et al.[44] The authors demonstrated that nylon 6,6 could be applied as a substrate-independent adhesion layer for subsequent brush grafting of oligo(ethyleneglycol)-methacrylates and carboxybetaine acrylamides by ATRP.

Beside the above-mentioned CM-dextran, OEG/PEG derivatives, and polycarboxybetain, poly(vinyl alcohol),[46] polycarboxylates,[47] and naturally occurring polymers such as cellulose[48] have also been applied as protein resistant matrices. However, as blocking the reactive sites with BSA or casein is sufficient for most microtiter plate assays, these methods are still standardly used for rendering assays protein resistant.[36] Nevertheless, highly sensitive systems are needed in applications such as biosensors or protein microarrays particularly for point-of-care (POC) diagnostics.[49] Since POC diagnostics do not involve preparation of samples by laboratory staff, the most common samples are whole blood and other body fluids such as saliva or urine. Existing microtiter plate based assays may in some cases only be able to detect disease markers at concentrations that correspond to an advanced stage of a disease.[50] Therefore, improvement in the sensitivity of POC devices is a major concern. For a given cancer diagnosis marker, mainly two options exist for improving the sensitivity of POC devices. The first one is to improve the measuring technique itself or to develop even newer sensing devices. The second option would be to reduce the background signal by applying bioinert coatings.[51] One example for a commercial POC device applying a protein resistant matrix is an early detection system for cervical cancer, which is the second most common cancer in women (Figure 4). This so-called "papillo check" assay can detect PCR-amplified fragments of the high-risk papillomavirus (HPV) which constitutes the primary cause of almost all cervical cancers.[17] Hence, the "papillo check" nicely shows how bioinert matrices can significantly enhance the signal-to-noise ratio. Nevertheless, above-mentioned dextran coatings still represent the standard bioinert matrix for biosensor devices. However, dextran coatings are not always suitable, for example, when measuring lectins which specifically but weakly bind sugar moieties.[52]

Figure 4. Papillo check for early detection of the high-risk papillomavirus based on a DNA-hybridization-assay on microarrays. The biospecific (R1 = amino-PEG-linked oligonucleotides) and bioinert (R2 = hydroxyl-terminated PEG) groups on the grafted polymer brush can be controlled to obtain high signal-to-noise ratios. With kind permission of PolyAn GmbH, Berlin.

Primary packaging and handling of biopharmaceuticals

The development of recombinant therapeutic proteins has considerably increased over the last two decades. However, the manufacturing, shipping, and storing of protein-based drugs is challenging because of the likelihood of proteins to denature or aggregate. Although lyophilized proteins show an improved stability toward thermal denaturation, liquid formulations are easier to process, require less manipulation, and are therefore easier to administer.[53] However, proteins are more prone to chemical as well as physical stress factors in liquid formulations, which causes the proteins to denature, aggregate, or adsorb to the container surface during the commonly targeted two-year shelf life.[54] This may result in loss of therapeutic activity and even cause immunogenic[55,56] and allergic reactions.[57,58]

By far the most used primary containers for small-scale applications are Type I borosilicate glass vials. Despite their relative inertness and hydrophilic nature, glass surfaces may still adsorb large amounts of protein.[34,59] Frieß and coworkers have shown that the adsorption of IgG1 and IgG from

human serum (h-IgG) to type I glass vials is mainly driven by electrostatic interactions and is therefore highly dependent on the pH and ionic strength of the formulation.[60] They have shown that an increased charge difference between surface and protein correlates with an increased protein adsorption (Figure 5).
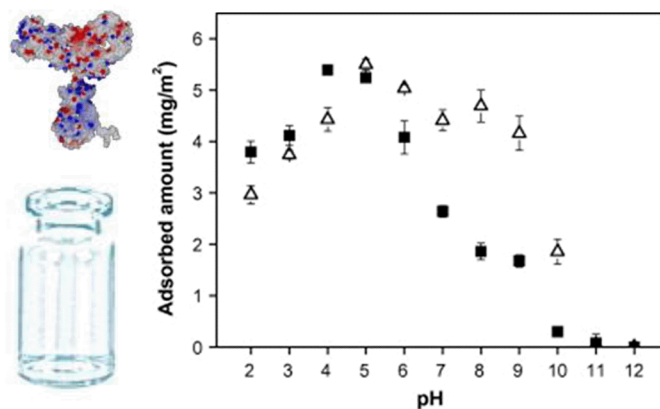

Figure 5. Adsorption profiles of monoclonal IgG1 (■) and pooled human IgG (△) in borosilicate glass vials depending on pH value. Reprinted with permission from J. Mathes, W. Frieß, *Eur. J. Pharm. Biopharm.* 2011, *78*, 239–247. Copyright 2011 Elsevier.[60]

An alternative to glass are a containers made of cyclic olefin polymers (COP) or cyclic olefin copolymers (COC). COP/COC containers are transparent, which allows for good visual inspection, and are considerably more lightweight and break-resistant than glass. Quadry et al. investigated the adsorption of low-concentration formulations (10 μg mL^{-1}) of two proteins (75, 20 kDa)[62] to COP and glass vials after 1 day of storage. For both protein formulations, the amount of adsorbed protein was considerably reduced in the case of the COP vials. In contrast, Mathes et al. found no significant decrease in single-protein adsorption experiments on COC vials compared to glass vials with IgG at concentrations of 1 mg mL^{-1}.[60] Indeed, they were able to show that the amount of adsorbed IgG1 over a range of ionic strengths and pH values even increased after 1 day of storage.

It has to be noted that issues associated with protein adsorption to container surfaces are generally overcome by the addition of excipients, like human serum albumin (HSA), sugars, or nonionic surfactants.[63-65] These excipients not only decrease the adsorption to the primary container but additionally increase the solubility and bulk stability and reduce the adsorption of the protein during the manufacturing process, especially when it is filtered and therefore exposed to large surface areas.[8] However, since HSA is obtained from pooled plasma, its use may implicate problems related to batch

variations or blood-borne pathogens,[65] whereas nonionic surfactants can increase oxidation and aggregation of the protein.[66] The use of sugars can be problematic as well due to hyperosmolality of the formulation.

Polymeric coatings can considerably reduce protein adsorption, and protein loss can be negligible (below 0.1%) in the case of high-dose formulations.[60,67] However, when labile proteins and low-dose formulations come in contact with container walls during reconstitution or when formulations are diluted for the administration, the protein loss due to adsorption is commonly quite significant. For example, McLeod et al. reported the adsorption-induced loss of factor VIII during storage in PVC containers.[68] Another example for an increased protein loss for highly diluted protein solutions is given by Quadry et al., who found a significant loss of up to 16% for protein concentrations of 10 μg mL^{-1} after storage for 24 h in glass vials.[62] Page et al. tested the adsorption from liquid formulations of rhIL-11 to glass vials at concentrations of 1 μg mL^{-1} and found a 40% loss of activity after storage for 3 h at room temperature.[69] This loss of activity was mainly caused by the adsorption to the glass surface.

Furthermore, when proteins are handled in diagnostics, low-concentration protein solutions are usually exposed to a variety of surfaces.[70] The accompanying protein loss can have a major impact on the outcome of a diagnostic assay. For example, Dixit et al. investigated the activity loss of human fetuin A (HFA) in BSA-treated and untreated PP tubes, respectively, at concentrations of 10 ng mL^{-1} with three different methods.[71] They were able to show that the HFA activity loss due to adsorption and conformational changes after 12 h of storage was above 60% in untreated PP tubes, whereas the loss was below 40% in BSA treated tubes. An alternative approach for rendering biomedical devices, protein resistant was chosen by Doran, who modified surfaces by physisorption of PEG and various Pluronic surfactants.[72] Both Pluronic and PEG coatings could significantly increase the recovery rate of mouse IgG1. Despite the promising results obtained from physisorption of BSA, PEG, or surfactants, covalent immobilization may offer several advantages. The displacement of adsorbed proteins by proteins with a higher surface affinity, the so-called Vroman effect, is well known and may in this context cause a loss of the sample protein due to an exchange with the previously adsorbed blocking agent. Vogler and coworkers demonstrated the Vromann effect by performing sequential adsorption experiments showing that albumin becomes replaced by IgG.[73] Physisorbed surfactants and PEG are also prone to detachment due to their good water solubility and may therefore contaminate the sample in the same way as albumin.

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Hence, the problem of protein analyte loss in diagnostic assays is still a problem that needs to be addressed, in particular in cases where a high purity of the sample is required or when measuring low abundant disease markers like in Alzheimer's disease.[74]

1.2 Marine biofouling

The large number of 4000 marine fouling organisms, that have been identified so far indicate the complexity of biofouling in marine environments. Some of the most common are bacteria,[75] diatoms,[76] algae,[77] mussels,[78] and barnacles.[13] Each of these species can adhere to solid surfaces via their own individual mechanism, which makes the development of antifouling coatings a complex task. One of the most prominent adhesion mechanisms is observed in blue mussels (*Mytilus edulis*), which has already been exploited for medical adhesives.[79] As soon as mussels find a place to filter sea water for plankton, they strongly attach to almost any kind of surface by applying several protein adhesives. These so-called mussel foot proteins or mussel adhesive proteins contain a large amount of dihydroxyphenylalanine (L-DOPA), a rare amino acid, that enables the formation of a highly cross-linked polymeric network. Whereas the composition of mussel adhesive proteins are well documented, barnacle adhesives are considerably less studied. It is known, however, that in contrast to mussel adhesive proteins and tubeworm cement, barnacle adhesive does not contain L-DOPA.[13] The absence of L-DOPA is noteworthy as barnacle cement represents the only permanent underwater adhesive of an invertebrate that lacks L-DOPA. Nevertheless, barnacle adhesion relies on the use of proteins. Instead of crosslinking via quinones as in the case of mussels, a crosslinking of proteins via disulfide bonds was assumed due to the high cysteine content.[80] An example for a non-calcareous fouling organism is the green alga ulva, which uses a glycoprotein adhesive to attach to solid surfaces.[81] The composition of the adhesive layer, however, is still poorly understood.[82] Another group of algae are diatoms, whose adhesive layers are mainly comprised of polysaccharides and proteins.[83]

At first sight, rendering ship hulls resistant against biofouling seems to be unrelated to the task of making biomedical devices bioinert. However, all of the above-mentioned fouling organisms use protein adhesives to settle on solid surfaces to some extent. Therefore, bioinert coatings such as hydrogels which have been found to perform well in biomedical applications can also be used to make surfaces resistant against marine biofouling. This correlation is reinforced by the fact that barnacle cement polymerization has been assumed to exhibit the same mechanism as blood clotting.[84]

As a result, hydrogel coatings were tested for marine biofouling in this work and will be discussed after describing the state of the art as well as future perspectives of antifouling strategies in marine environments.

1.2.1 Antifouling strategies

Biofouling represents a severe problem for a variety of marine industries and applications such as the shipping industry, aquaculture, ocean monitoring, marine platforms, and offshore rigs. In the shipping industry detrimental effects of biofouling largely result from an increase of the frictional drag, which in turn is caused by an increased surface roughness due to the attached organisms. This means higher fuel consumption and higher operational costs and a greater emission of green house gases.[18] Antifouling paints that prevent the attachment of fouling organism save about 60 billion US dollars for fuel annually, not including the cost savings for dry-dock hire, off-hire, hull-cleaning and paint application.[85] In contrast, the effects of biofouling in aquaculture are very diverse, ranging from aesthetic considerations due to growth of calcareous organisms on shell fish, competition for food and space, restriction of water exchange due to net occlusion, and increased material abrasion.[86] The herewith associated annual costs in the US alone are estimated at 1.5 to 3 billion US dollars. The attachment of fouling organisms also limits the use of marine underwater sensors such as optical windows of transmissiometers or cameras, membranes of chemical sensors, and electrodes of pH-meters. The consequences are decreased data quality and increased maintenance frequencies of unmanned oceanographic platforms. As fouling related costs in shipping industry considerably exceed the costs in other marine industries, most of the antifouling strategies are tailored to protect ship vessels from biofouling. Therefore, the following chapters will first discuss types of antifouling coatings for ship vessels, namely ones that function by releasing biocides which kill settled organisms or by minimizing the adhesion of organisms and thereby facilitating their removal by the water flow.[87]

Biocidal antifouling coatings

Since the approval of tributyl tin (TBT) based self-polishing coatings in the mid-1960s, TBT has been the most frequently applied biocide in commercial shipping. After its ban in 2008 by the International Maritime Organization of the UN, copper and copper based compounds have evolved as the main

biocides in antifouling paints.[16] Most commonly copper is incorporated in the paint in pigment form as cuprous oxide (Cu_2O), giving the ship hulls their typical reddish and brownish colors.[88] Currently available commercial biocidal coatings can be subdivided into three categories (Figure 6):[18,87,88]

(i) Contact leaching paints (CLP) comprising insoluble high molecular weight binders such as vinyl or acryl copolymers and water soluble biocides. Contact with sea water dissolves the biocides thereby creating pores that in turn get filled with sea water which dissolves further biocides. As the water needs to penetrate deeper and deeper into the paint to dissolve biocides, the release rate of biocides exponentially decreases over time. Therefore, the lifetime of CLP is generally limited to one year. Another disadvantage is the increase in surface roughness due to the creation of pores.

(ii) Controlled depletion polymer (CDP) paints partially contain soluble binders which simultaneously dissolve with the biocide, thereby creating a mechanically weak porous binder network that can be ablated in layers by the water flow. The lifetime of CDP is around three years.

(iii) Self-polishing copolymer (SPC) paints are based on water insoluble acrylic copolymers containing carboxylate side groups bonded to copper or zinc ions. Ion exchange of the metal ion cleaves the copper or zinc ions from the copolymer which renders the polymer water soluble. As the polymer binder is water insoluble, the sea water cannot penetrate into the paint layer thus causing a layer by layer ablation of the paint and leading to a continuous surface renewal. An advantage of this self-polishing effect is the resulting smooth surface associated with lower fuel consumptions. Lifespans of up to five years can be achieved.
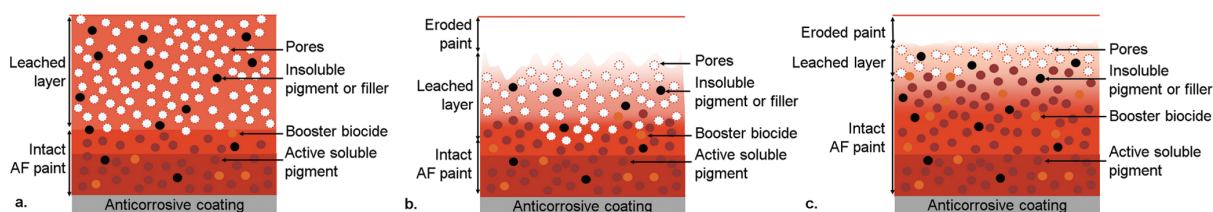

Figure 6. Schematic illustration of main biocidal antifouling paint classes. (a) Contact leaching paint (CLP); (b) controlled depletion polymer (CDP) paint; (c) Self-polishing copolymer (SPC) paint. Reprinted with permission from M. Lejars, A. Margaillan, C. Bressy, *Chem. Rev.* 2012, *112*, 4347–4390. Copyright 2012 American Chemical Society.[18]

Driven by the large environmental impact of the biocidal release by antifouling paints, the three-fold increase of the copper price in the last ten years and the increased restrictive legislation for the approval of new biocides has led to the development of more economically friendly paints that do not apply biocides.[89]

Non-biocidal coatings

The group with the second largest market share among commercial antifouling paints are fouling release coatings (FRCs). This approach is based on reducing the adhesion strength of fouling organisms. To date, only two classes of polymers have found to exhibit a good fouling release performance, namely, silicones and fluoropolymers.[88] On these smooth hydrophobic surfaces, barnacles can hardly attach. Due to the reduced surface wettability, a cavity is formed below the barnacle which makes the curing of the adhesive impossible. Therefore, ship hulls coated with FRCs can easily be cleaned with brushes underwater or by shear forces during ship movement. Whereas silicone based FRCs are already a commercial success, fluoropolymer based coatings are still niche products. In general, silicone FRCs consist of cross-linked polydimethylsiloxane (PDMS) which contain silicon oil additives such as phenylmethyl siloxanes that enhance the non-stick properties of the coating.[90] Silicon based FRCs are supplied as a two- or three-component system, containing the silicone precursor, catalyst, and crosslinker, respectively.[18] Two distinct crosslinking chemistries are used to apply the coating to the ship hull, namely, condensation or hydrosilylation.[91] FRCs exhibit lifetimes of 5 to 10 years.

According to an estimation by Eliasson, the costs per m^2 and year including costs for paint, off-hire, dry dock time, application, hull cleaning, and surface preparation are less for FRCs than for all biocidal antifouling paints.[92] However, it has to be considered that these calculations are based on an estimated lifetime of FRCs of ten years, whereas the lifetimes specified by manufacturers are often only five years. Calculations based on a lifetime of five years show that overall application costs of FRCs exceed the costs of biocidal paints by a factor of 1.5. These calculations, do not take into account the cost savings that result from reduced fuel consumption due to the decreased surface roughness of FRCs. Hempel, for example, guarantees for its third generation FRC a reduction in first-year fuel consumption of 4-8% depending on the vessel type, which does make these coatings less expensive than biocide based paints.[93] Since information about prices from manufacturers is not really accessible, these considerations are only based on estimations and therefore need to be

considered with caution. Market shares of FRCs may also give an indication of the overall cost. In commercial shipping the total FRC sales is above 10%, whereas the rest of the market belongs to biocidal paints.[94] In contrast, total sales of FRCs in the yacht sector of less than 1% show the greatest drawback of FRCs, namely, that their antifouling performance depends on the ship velocity. But their performance has considerably improved over the years. Whereas the first commercial FRCs were limited to high-speed vessels (> 30 knots), the current best performing FRCs work on ships that travel at 10 knots or less (Figure 7).[18] Since ships in the yacht sector spend a lot of time under static conditions, the ultimate goal is to develop coatings that effectively self-clean without any ship movement. Furthermore, effective prevention of biofouling under static conditions is also highly relevant in aquaculture and underwater instruments.[86,95] One approach to improve the antifouling performance of FRCs is to use hybrid systems which incorporate additional components such as hydrogels and nanofillers.
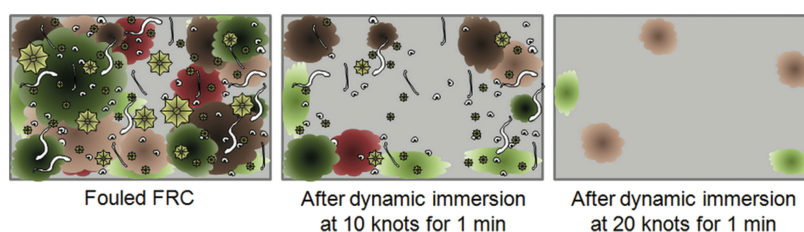

Figure 7. Schematic illustration of fouling release behavior. Reprinted with permission from M. Lejars, A. Margaillan, C. Bressy, *Chem. Rev.* 2012, *112*, 4347–4390. Copyright 2012 American Chemical Society.[18]

As in this work the development of hydrogel based coatings for use in underwater applications is described, the following chapters will discuss hydrogel based coatings that prevent fouling under static conditions.

Hydrogel based coatings

Even when calcareous biofouling is completely suppressed, slime film formation alone can cause an increased fuel consumption of 21%.[96] As FRCs are known to have difficulty preventing the formation of slime layers containing diatoms and bacteria, fabrication of hybrid systems composed of PDMS and a hydrogel, which are known to have a good resistance against bacteria and diatoms is an obvious approach. To date, one of the best performing FRCs was introduced by Hempel and is based on a PDMS binder incorporating pendant hydrophilic OEG/ PEG moieties (Figure 8).[97] This system shows good nonfouling performance down to a ship velocity of 8 knots. The use of hydrogels to prevent marine biofouling has also been addressed by academic research. Cowling and coworkers reported on

the antifouling properties of poly-2-hydroxyethyl methacrylate (PHEMA) based hydrogels against two algal species compared to acrylic and glass surfaces.[98] Whereas the adhesion of *Melosira nummuloides* on PHEMA hydrogels was considerably reduced compared to acrylic and glass surfaces, adhesion of *Enteromorpha intestinalis* link zoospores only slightly decreased. When exposed to the ocean in April in the northern hemisphere, PHEMA hydrogels slightly fouled with diatoms and algae within a period of three weeks, whereas exposure in February resulted in visually clean surfaces after four weeks. Due to their poor mechanical strength, most hydrogels are limited in their use to short periods. Gong and coworkers tried to address this problem by increasing the mechanical strength by development of crosslinked hydrogels.[99] They reported on the antifouling properties and long-term stability of physically cross-linked PVA and interlocked poly(2-acrylamide-2-methyl-1-propanesulfonate) (PAMPS)/ poly(acrylamide) (PAAm) hydrogels in a marine environment. After immersion periods of one year, no physical damage could be observed. Furthermore, both gels showed an improved antifouling performance by a factor of five compared to unmodified polyethylene (PE) substrates. Hence, physical properties of hydrogels could be adjusted to fulfill the criteria that have to be met in static marine environments. Another article by Gong and coworkers demonstrated that hydrogels including PVA, PAAm, poly(sodium p-styrene sulfonate) (PNaSS), poly (N, N-dimethylaminopropylacrylamide, methylchloride quarternary) (PDMAPAA-Q) not only resist the adsorption of algae and diatoms, but also the adsorption of calcareous fouling organisms such as barnacles.[100] The authors presented an extensive study on a broad range of neutral and charged hydrogels with respect to their ability to resist the adsorption of barnacle cyprids (*Balanus amphitrite*). While all the hydrogels significantly reduced the settlement of cyprids compared to a PS surface no correlation could be observed between the charge of the hydrogel and the degree of fouling. The authors further argued that charge effects might be less important in marine environments due to the screening of the charge because of the high ionic strength of seawater. Two approaches that included PEG as hydrogel coatings on a laboratory scale were presented by Liedberg and coworkers and Hult and coworkers, respectively.[101,102] Liedberg and coworkers coated glass substrates with PEG hydrogels in a two-step procedure via free-radical polymerization of PEG-methacrylates under inert gas atmosphere. The coatings showed good nonfouling behavior against microorganisms including algal zoospores, diatoms, and barnacle cyprids. Hult and coworkers also applied a two-step coating procedure using thiol and allyl endfunctionalized PEG with varying chain lengths to form crosslinked hydrogels under UV light via thiol-ene chemistry. Adsorption tests of BSA, marine bacteria (*Cobetia marina*), and diatoms (*Amphora coffeaeformis*) showed an improved nonfouling performance with longer PEG chains.
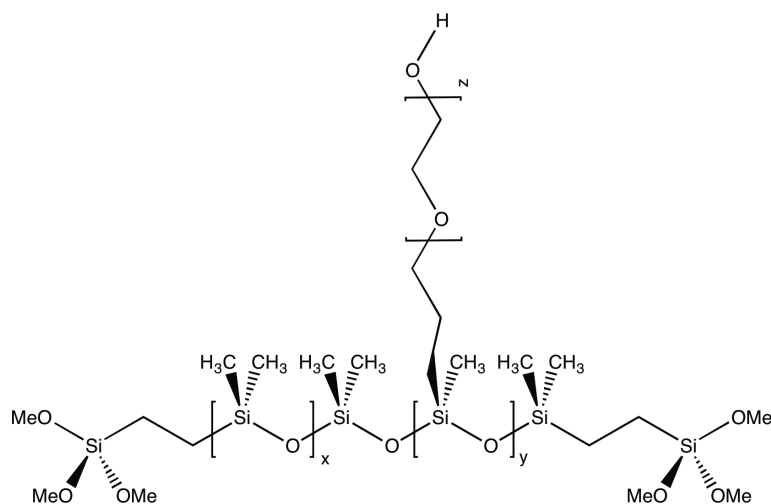
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Figure 8. PEG-PDMS based binder-precursor for a fouling release coating (FRC) patented by the Hempel A/S.[97]

Most of the above-mentioned hydrogel based coatings have only been applied on a laboratory scale. Therefore, further research on the development of cost efficient production methods, a practical application of the coatings, and increased mechanical strength is necessary to replace the current antifouling systems for optical sensors, which mainly rely on three distinctive strategies: (i) mechanic cleaning using automatic wiping or scrubbing devices, (ii) biocidal release coatings comparable to the ones applied in the shipping sector, and (iii) controlled generation of biocides using automatic electro-chlorination or acid dispensing devices.

1.3   Switchable surfaces

1.3.1   Tissue engineering

Organ transplantation is considerably limited by the available amount of organs and tissue needed to treat all the patients on transplant waiting lists.[103] Hence, every year many people die waiting for an organ donation. Furthermore, allogeneic transplants can cause problems with the host immune system, leading to rejection or destruction of the donor tissue. The emerging field of tissue engineering tries to overcome these problems by creating perfectly tailored tissues *in vitro*. Therefore, cells are cultured *in vitro* on an adequate scaffold that enables transfer of the artificially grown implant to the host tissue. Although this technology holds great promise, today only very simple tissues such as skin and cartilage are commercially available.[104] Two major problems currently exist in the fabrication of more

complex tissue. The first problem is the inability to create a vascular network that can connect to the vascular system of the host, which is necessary for the supply of nutrients and oxygen after implantation. As the diffusion limit of oxygen is around 100-200 μm, only tissue thinner than this limit can be sufficiently supplied with oxygen.[105] Therefore, only tissues such as cornea and skin can be supplied with nutrients and oxygen by diffusion from existing blood vessels in the host tissue.[104] The second problem is the spatial organization of the cells in tissue that resembles the original tissue with respect to morphology and physiology.[103] Therefore, scaffolds are needed for complex tissue fabrication that serve as templates for the tissue formation by directing the organization of the cells via interactions with the scaffold, which can be adjusted in terms of topography, mechanical properties, and growth factors.[106] The fact that all of the engineered tissues now being used in clinics are soft tissue applications with two-dimensional scaffolds not only reveals the difficulties in creating sufficient vascularization, but also in developing suitable three-dimensional scaffolds for the construction of complex tissue.

For the fabrication of soft tissue such as endothelium and epithelium, scaffolds that mimic the extracellular matrix (ECM) can be dispensed, since cells secret their own ECM during proliferation. However, as almost all cells need to attach to a solid surface to proliferate, they need to be detached after proliferation. Commonly, this is achieved by harvesting cells via mechanical dissociation of the cell sheet or proteolytic cleavage of the ECM. For obvious reasons, these methods cannot be applied for tissue fabrication. To control the adhesion and detachment of cells, less harmful methods have been developed that preserve the ECM.[107] These techniques utilize an external stimulus to trigger the release of cell sheets which enables subsequent transfer of undisrupted tissue. For example, Zambelli and coworkers have developed a matrix based on polyelectrolyte multilayers that can be used for culturing cells and then disrupted by adding ferrocyanide to release confluent cell sheets.[108] Therefore, they alternately deposited poly(L-lysine) (PLL) and hyaluronic acid (HA) via a layer-by-layer (LBL) approach with a top layer of fibronectin to enhance cell adhesion. The advantage of this approach is its simplicity because only natural available polymers were used and the fact that the coating can be applied to almost any substrate. The disadvantages would be both the need for fibronectin as artificial ECM, since coatings made of PLL/HA only exhibit weak cell adhesion, and the presence of polyelectrolytes, in particular of cell toxic poly(L-lysine).[109] Vörös and coworkers have shown that confluently grown cell sheets on LBL films of PLL/HA and PLL/poly(L-glutamic acid) (PGA) even spontaneously detached without any external trigger.[110] The authors ascribed this observation to the weak adhesion of the cells to LBL. These authors also described another approach based on polyelectrolytes.[110,111] In contrast to the afore-mentioned approach, the dissolution of the polymeric

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matrix was not achieved by adding salt but by electrochemical polarization of a conducting substrate. To realize their idea, the authors synthesized an RGD modified poly(L-lysine)-*graft*-poly(ethylene glycol) (PLL-*g*-PEG/PEG-RGD) polymer. This polycation-backbone enabled immobilization on oppositely charged surfaces, namely, indium tin oxide (ITO) and specific adhesion of cells via the integrin binding RGD moieties. After cells were grown to confluence on PLL-*g*-PEG/PEG-RGD coated ITO substrates, the cell sheet could be harvested by applying a positive potential. Drawbacks of this method, however, are the presence of polycation-copolymers and potential binding of the copolymers to detached cells via the RGD motif.

Honda and coworkers detached cell sheets based on the use of magnetite cationic liposomes (MCL).[112] To promote cell adhesion the authors coupled an RGD-motif containing peptide to the MCLs. These RGD-MCLs were added were added prior to cell culturing to a culture substrate comprising a covalently bound hydrogel and fixed by placing a magnet underneath. After growing to confluence, the cell sheets were detached by removing the magnet. Due to the binding of the cell sheet to the RGD-motif, the detached cell sheets contained magnetite nanoparticles, which could be used to transfer the cell sheet to another substrate using a magnet. The authors claimed that the amount of MCLs in the cell sheets was considerably below the toxic level. However, further studies are needed regarding the fate and toxicity of the remaining particles. Another cell-sheet engineering approach is based on the enzymatic digestion of the substrate.[113] Therefore, Nagai and coworkers used salmon atelocollagen (SAC) as the matrix and collagenase to digest the SAC in order to harvest confluent cell sheets. One drawback of this method is the long digestion time of the collagen gel of 2 h. Pasparakis and coworkers reported on a laser-induced cell detachment using photodegradable substrates.[114] The authors applied two polymers comprising ketal or acetal repeating units as photodegradable substrates to culture fibroblasts. After cell seeding and growth, the cells were harvested by a layer-by-layer polymer ablation at a wavelength of 193 nm. The authors claimed that the cells were not directly exposed to the laser beam. Beside all the above-mentioned methods for cell sheet engineering, whose development is still in its early stages, a promising approach applying thermoresponsive substrates has evolved over the last two decades. This method has been tested for the fabrication of tissues such as myocardial tissue[115] and autologous epithelial cell sheets used for corneal reconstruction,[116] both of which are in preclinical or clinical trials, respectively. Thermoresponsive cell sheet engineering has added the advantage that the polymeric layer is covalently bound to the surface throughout the entire cell-sheet fabrication process. This means that no polymers or small molecular cleavage products can be internalized by the cells or bound to the ECM after cell sheet detachment.

In this approach covalently grafted thermoresponsive polymer based coatings serve as substrate for a controlled cell adhesion and detachment. Thermoresponsive polymers exhibit a discontinuous solubility with temperature. Depending on whether the polymer is soluble above or below a respective temperature, these temperatures are called upper critical solution temperature (UCST) and lower critical solution temperature (LCST), respectively. The most common explanation for aqueous LCST systems is based on a negative entropy (ΔS) term that renders the polymer-water interaction unfavorable above a certain threshold temperature according to the relation $\Delta G = \Delta H - T\Delta S$.[117] This temperature induced phase separation can be described by the hydrophobic effect that explains the poor solubility of hydrocarbons in water. When mixing hydrocarbons and water, hydrocarbon-water interactions are at the expense of more favorable water-water interactions. In order to compensate for the lost hydrogen bonds, the remaining water-water interactions of the hydrocarbon binding water molecules are strengthened, which makes the water structure more rigid or "ice-like" and thus entropically unfavorable. In the case of a polymer with an LCST, the negative entropy outweighs the enthalpy at a certain temperature and results in a phase separation that is associated with an entropically favorable release of previously polymer-bound water molecules. Further on the term "thermoresponsive" will only be used for polymers with an LCST.

When covalently grafted to a surface, the release of water from a highly water swollen thermoresponsive hydrogel associated with a collapse of the polymer chains renders the surface more rigid, more hydrophobic, and more adsorptive to proteins. This technique enables to switch between a protein repellent and a protein adsorptive state and, since cell adhesion is generally mediated by an adsorbed layer of plasma proteins this technique also enables to control the adhesion and detachment of cells. Whereas above the respective switching temperature of the polymeric layer the polymer exists in its collapsed form on which cells can easily adhere and proliferate, below the switching temperature the polymer chains are in a more extended conformation causing a detachment of the cell sheets (Figure 9).
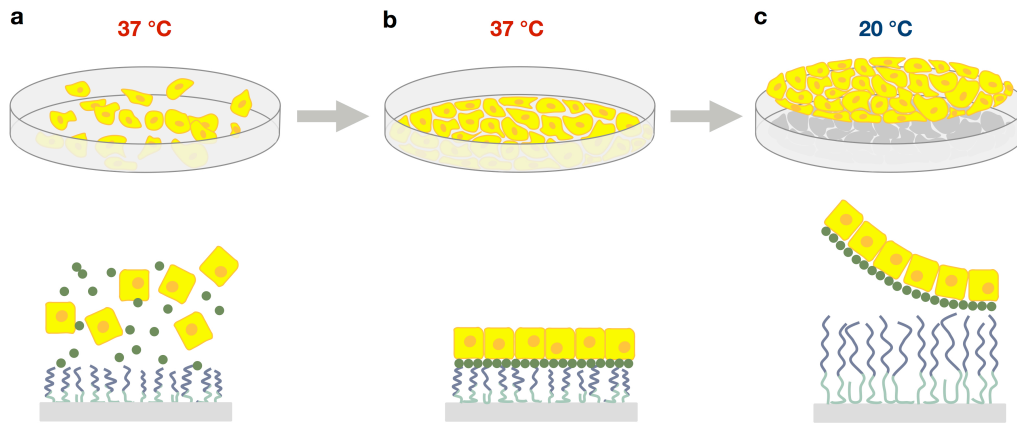
Figure 9. Cell culturing on thermoresponsive substrates. (a) Cells are seeded on a substrate coated with thermoresponsive polymer. The grafted polymer chains are in their collapsed form above the respective switching temperature. (b) Cells are grown to confluence at 37 °C. (c) Confluent cell sheets spontaneously detach when decreasing the ambient temperature below the respective switching temperature.

The by far most extensively studied thermoresponsive polymer in cell sheet engineering is poly(*N*-isopropyl acrylamide) (PNIPAM), which has been used to fabricate tissue that has already been tested in clinical trials. In 2004 Okano and coworkers described the fabrication of autologous oral mucosal epithelial-cell sheets and their use in corneal reconstruction.[116] Therefore, mucosal epithelial cells were collected from a patient's oral cavity and seeded on PNIPAM coated dishes. After two weeks of cultivation, multilayered epithelial cell sheets could be detached by reducing the temperature of the medium to 20 °C for 30 minutes. Subsequently, the fabricated epithelial tissue was implanted into the patient's eye. In a follow-up study performed after 14 months all corneal surfaces were still transparent. Furthermore, this approach has also been used for the fabrication of more complex tissue composed of heterogeneous cells (Figure 10).[118] For example, Okano and coworkers fabricated three-dimensional tissue comprising a vascular network by stacking fibroblast cell sheets and micro-patterned endothelial cells as vascular precursors harvested from PNIPAM substrates.[119] After assembly and 5 days of further tissue culture the formation of microvascular-like networks could be observed.
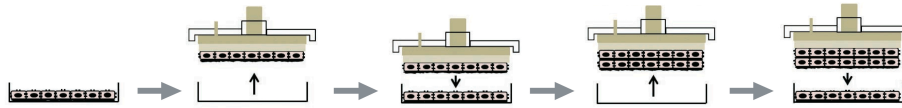
Figure 10. Thermoresponsive cell sheet engineering using Okano's method. Fabrication of three-dimensional tissue by layering cell sheets that were cultured on thermoresponsive culture dishes. Cell sheets are recovered and stacked by applying a hydrogel coated plunger-like manipulator. Reprinted with permission from Y. Haraguchi, T. Shimizu, T. Sasagawa, H. Sekine, K. Sakaguchi, T. Kikuchi, W. Sekine, S. Sekiya, M. Yamato, M. Umezu, et al. *Nat. Protoc.* 2012, *7*, 850–858. Copyright 2012 Nature Publishing Group.[120]

Beside PNIPAM various other thermoresponsive polymers have been applied in cell-sheet engineering. In 2006 Chen and coworkers prepared thermoresponsive hydrogels from blends of methylcellulose (MC) and distinct salts.[121] After deposition of aqueous collagen on MC coated culture dishes in order to promote cell adhesion, cells were grown to confluence and subsequently harvested by reducing the surrounding temperature to 20 °C. Another cellulose based approach was described by Yue and coworkers.[122] Therefore, hydroxypropyl cellulose (HPC) was modified with methacrylic anhydride to adjust the LCST to 37 °C in deionized water. Cell sheets of African green monkey kidney cells detached spontaneously after reducing the temperature to 4 °C. Other examples are given by Chan and coworkers who applied hydroxybutyl chitosan to study the detachment dynamics of singular smooth muscle cells (SMC)[123] and by Lutz and coworkers who applied PEG based surfaces[124] for controlled detachment of single cells.

Finding the ideal thermoresponsive surface that provides suitable culture conditions as well as high attachment and detachments rates is a challenging task, mainly because of the individual requirements for each cell type. Therefore, various systems need to be developed and compared to existing ones. Of particular interest are systems with adjustable properties, as they can be easily tailored to individual cell types.

2 Objectives

The chemical and physicochemical properties of glycerol based polymers can be adjusted by incorporating additional monomers or by fully or partially modifying the large amounts of the present hydroxyl groups after polymerization. This enables the development of a wide variety of PG based coatings that can serve different functions without compromising the excellent non-fouling properties of PG. Within this work, polyglycerol based nonfouling, and switchable coatings will be synthesized and evaluated for biomedical and marine applications.

In the first project, several polyglycerol based coatings will be evaluated for their ability to render borosilicate glass vials protein resistant and thus increase the recovery rate of the proteins stored therein.

In the second project, a functional coating for biosensors will be developed for application on gold surfaces. For this purpose, functional groups that will allow subsequent binding of biomolecules and a grafting moiety will be incorporated into dPG to enable immobilization on gold. Additionally, the above-mentioned coated glass vials will be evaluated in this project as containers for handling low concentration protein solutions.

In the third project, a new coating for glass will be prepared that can be easily applied to large surfaces and resist marine biofouling.

In the fourth project, a thermoresponsive coating for controlled cell-sheet detachment will be developed. Therefore, two commercially available glycidol based monomers will be copolymerized with varying ratios to adjust the switching temperature of the resulting polymer to a suitable value. Subsequently these coatings will be evaluated with respect to their temperature-dependent protein resistance and their ability to switch between a cell adhesive and a cell repellent state.

3 Publications and manuscripts

3.1 Polyglycerol coatings of glass vials for protein resistance

Kerstin Höger, Tobias Becherer, Qiang Wei, Rainer Haag, Wolfgang Frieß, Sarah Küchler[*]
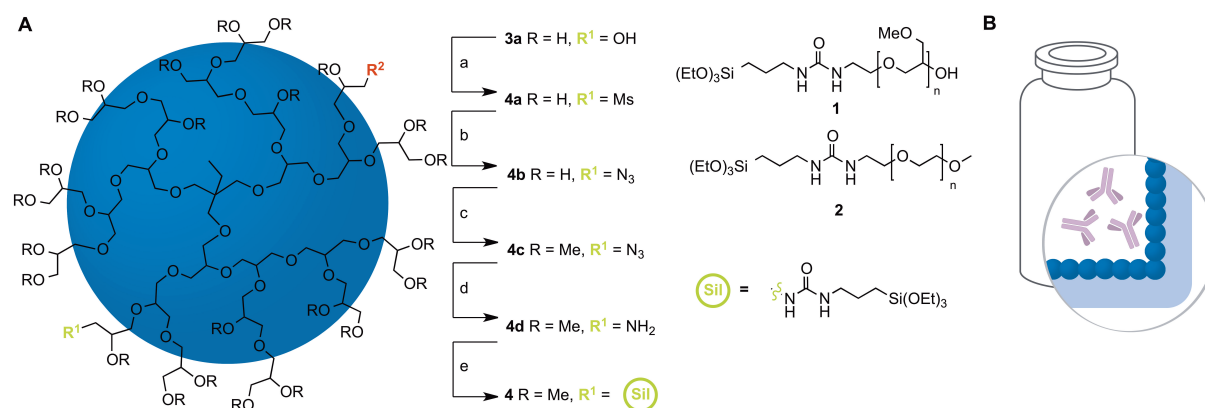
This chapter was published in:

Contribution:

Synthesis, characterization, and surface analysis by QCM-D were performed by the author. The coating was applied by the author and Q. Wei. Protein stability and adsorption experiments were performed by K. Höger.

In the following publication a different notation for the glycerol based polymers was used:
dPG = HPG(OH); dPG(OMe) = HPG(OMe); lPG(OMe) = LPG(OMe)

[†] contributed equally

3.2 Polyglycerol based coatings to reduce non-specific protein adsorption in sample vials and on SPR sensors

Tobias Becherer,[*] Christian Grunewald, Vivienne Engelschalt, Gerhard Multhaup, Thomas Risse, Rainer Haag
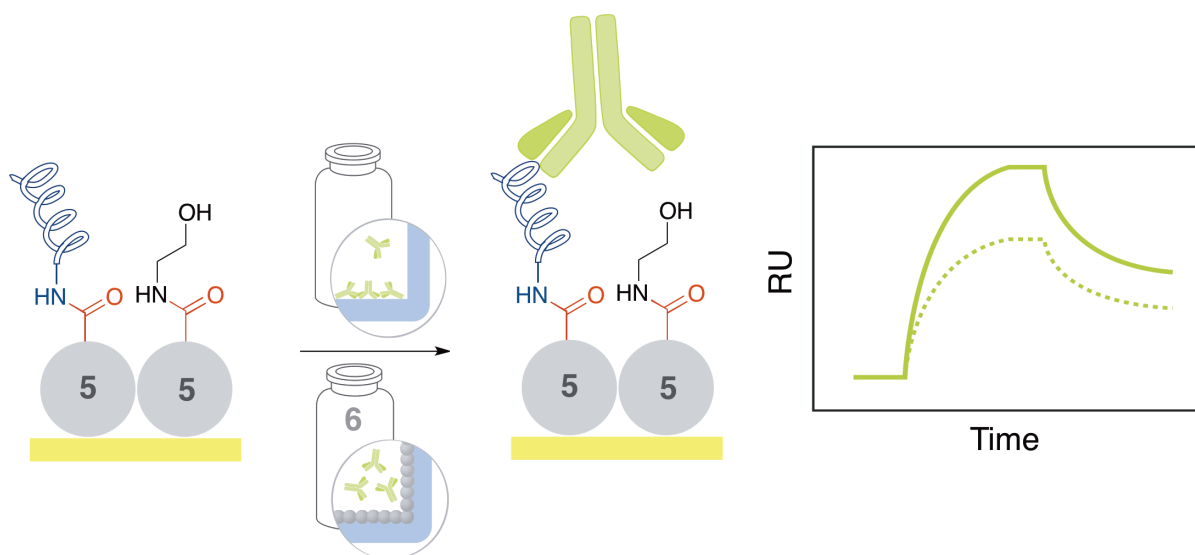
Contribution:

Synthesis, characterization, coating, and surface studies by SPR were performed by the author. Surface studies by QCM-D were performed by the author and C. Grunewald. Antibodies were produced by V. Engelschalt.

3.3 Fast and Easily Applicable Glycerol Based Nonfouling Spray-coating

Tobias Becherer, Matheus Vieira Nascimento, Julian Sindram, Paul-Ludwig Michael Noeske, Qiang Wei, Rainer Haag,[*] Ingo Grunwald[*]

Contribution:

Synthesis was performed by the author and J. Sindram. Characterization, coating, ellipsometry, contact angle measurements and surface studies by QCM-D were performed by the author. Blue mussel and algae adhesion experiments were performed by I. Grunwald and the author. XPS-measurements were performed by P.-L. M. Noeske. Bacteria adhesion experiments were performed by M. V. Nascimento.

3.4   Switchable, biocompatible surfaces based on glycerol copolymers

Marie Weinhart,[*] Tobias Becherer, Rainer Haag

This chapter was published in:

Contribution:

Synthesis, characterization, surface immobilization, SPR studies, and cell experiments were performed by the author and M. Weinhart.

3.5 In-Depth Analysis of Switchable Glycerol Based Polymeric Coatings for Cell Sheet Engineering

Tobias Becherer, Silke Heinen, Qiang Wei, Rainer Haag, Marie Weinhart[*]

Contribution:

Synthesis, characterization, surface immobilization, SPR studies, and DLS experiments were performed by the author. Cell experiments were performed by Q. Wei.

4   Summary and conclusion

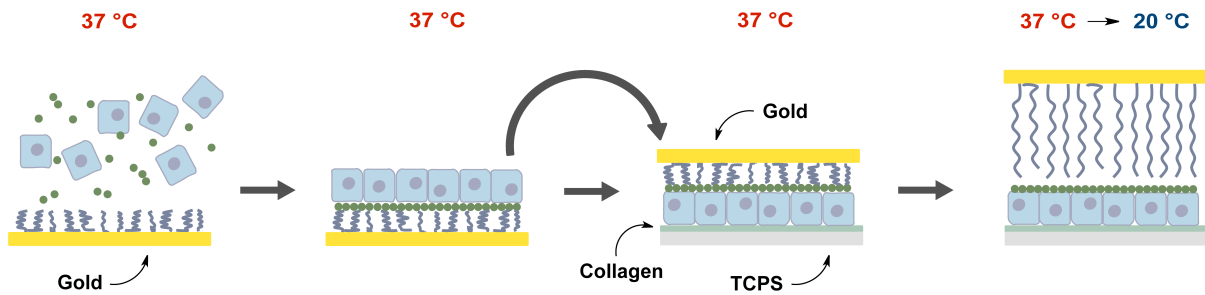In this work, the preparation and application of glycerol based polymers as coatings were described. The coatings were classified into passive and responsive materials. Whereas the coatings of the first group were developed to passivate materials and thus reduce biofouling, the fouling behavior of the second group could be controlled by adjusting the ambient temperature. The first group was further subdivided into nonfouling surfaces for biomedical applications and on the other side marine applications. For biomedical applications, dendritic polyglycerol (dPG), methoxylated dPG (dPG(OMe)), and linear methoxylated polyglycerol (lPG(OMe)) were evaluated as coatings for glass vials that serve as primary packaging material for therapeutically relevant proteins at high concentrations ($\geq$ 1 mg mL^{-1}). To enable a practical application of the coatings to glass vials at low temperatures and without the use of inert gas, the previously published application procedure was modified accordingly. All coated glass surfaces showed a significantly reduced protein adsorption as compared to unmodified glass, even after heat or autoclaving sterilization. dPG based coatings were particularly found to exhibit superior stability against the tested and industrially relevant sterilization methods. Further stability tests of these siloxane grafted dPG coatings revealed their susceptibility to hydrolysis under alkaline conditions but also their long-term stability under storage-relevant conditions of at least three months.

dPG was further evaluated as a coating for glass containers of low concentration protein solutions where the percentage loss due to adsorption was considerably higher than for the high concentrations that are typically used for storing therapeutic protein solutions. By applying dPG(OMe) coatings to sample vials used as container for protein solutions in an SPR based immunoassay, it was demonstrated that, especially at low protein concentrations, dPG(OMe) could considerably reduce the percentage loss of proteins due to nonspecific adsorption. Therefore, a monoclonal antibody (mAb) against a surface immobilized peptide involved in Alzheimer's disease (amyloid beta 1-40), was applied as an analyte. The mAb was stored in uncoated and dPG(OMe) coated sample vials, respectively, prior to its use in the SPR based immunoassay. The considerably decreased signal in the SPR assay for the analyte from uncoated vials compared to the analyte from coated vials (23% signal loss at the lowest concentration of 1 μg mL^{-1}) showed that protein resistant coatings are essential for handling low-concentration protein solutions in protein based assays. Therefore, these coatings offer an excellent alternative to typically applied physisorbed blocking agents like albumin.

For the above-described immunoassay, carboxymethyl-dPG (CM-dPG) was synthesized as an immobilization matrix for amyloid beta 1-40 on gold sensors. Therefore, CM-dPG had to fulfill two functions: rendering the surface resistant against nonspecific adsorption of proteins and second enable the covalent immobilization of peptides. Adsorption experiments with fibrinogen have shown that incorporation of carboxymethyl groups into dPG caused a two-fold increase in the nonspecific adsorption. Nevertheless, the amount of adsorbed fibrinogen was still reduced by more than 99% compared to an uncoated gold surface, and by a factor of around six compared to a commercially available CM-dextran coated sensor. To test whether CM-dPG can be used to immobilize biomolecules, amyloid beta 1-40 was covalently bound to the matrix and successfully applied in the above-mentioned immunoassay to measure the binding interaction with the mAb (anti-Aβ1-40) at various concentrations.

Furthermore, a linear glycerol based copolymer was tested for its ability to prevent various fouling marine organisms from settling on glass. To facilitate the application of this coating to large substrates, the coating was applied by spraying, contrary to the above-mentioned coatings, which were all applied by immersion of the substrate. It was demonstrated that the adsorption of fibrinogen as well as the settlement of marine bacteria (< 1%), green algae (< 3%) and blue mussels (< 15%) could be considerably reduced compared to uncoated glass. Additionally, it was shown that the coating even outperformed polytetrafluoroethylene (PTFE) with respect to mussel adhesion. The coating durability is limited to some weeks when exposed in marine conditions.

Moreover, a responsive polymeric coating was prepared that enabled to control the adhesion of cells by the ambient temperature. Therefore, a series of linear glycerol based thermoresponsive copolymers was built from two different comonomers and contained a terminal thiol group. While the comonomers allowed one to adjust the switching temperature, the incorporation of a terminal thiol group enabled the immobilization to gold substrates. To access the switching temperature of these polymers when confined to a surface, the hydrodynamic radii of correspondingly coated gold nanoparticles were determined as a function of time. These results demonstrated that the switching temperature of the copolymers decreased upon grafting to a surface. It was also shown that all tested coatings exhibited an excellent protein resistant behavior below and a strong protein adsorptive behavior above the respective switching temperature. Finally, thermoresponsive coatings were successfully applied as culture substrates. Confluent cell sheets that were grown on thermoresponsive substrates, subsequently transferred to collagen coated culture dishes and further cultivated, showed excellent cell viability.

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5 Outlook

In order to sufficiently prevent protein loss due to adsorption, in particular when handling low-concentration protein solutions, it is necessary to extend the protein resistant coatings to other biomedical relevant materials such as polypropylene and polyethylene. The fact that polyolefins do not exhibit reactive functional groups further complicates the modification procedure, which in turn makes it a challenging task to find a cost-efficient procedure. Furthermore, for the coatings to be applicable in whole blood assays, it has to be shown that they can resist complex protein mixtures such as blood.

In the case of the marine nonfouling coatings, the hydrolytic stability needs to be further improved by shielding the siloxane anchor points from the seawater. This could be achieved by coatings consisting of a block copolymer with a water insoluble lower part and a hydrophilic upper part facing the water interface. Such coatings could, on the one hand, prevent hydrolysis of the siloxane bonds and, on the other, render the surface nonfouling. Alternatively, the overall hydrophobicity of the polymer could be increased. One way to increase the hydrophobicity without compromising the nonfouling properties would be to incorporate latent hydroxyl groups, which are protected by a hydrophobic ester group. Hydrolysis of these ester groups upon contact with seawater would cleave the hydrophobic residue and thus render the upper layer of the coating nonfouling and, at the same time, slow down the water penetration. These kinds of polymers with a latent nonfouling behavior might also allow one to include larger amounts of hydrogels into FRCs before the PDMS binder and the hydrogel phases separate and additionally with less impact on the mechanical strength.

For further testing the applicability of thermoresponsive polymers, the coating procedure needs to be extended to more relevant materials such as polystyrene. Furthermore, it has to be demonstrated that all kinds of relevant cells, in particular, primary cells can be grown and detached from these thermoresponsive substrates. Comparative studies with alternative systems with respect to biocompatibility, cell attachment, and detachment kinetics as well as cell morphology need to be performed in the future. Finally, the applicability of cell sheet engineering has to be evaluated in comparison to other tissue engineering methods.

6   Zusammenfassung

In dieser Arbeit wurde die Herstellung und Anwendung glycerol basierter Polymere als Beschichtungen beschrieben. Die Beschichtungen wurden in passive und responsive Materialien unterteilt. Während die Beschichtungen der ersten Gruppe dazu entwickelten wurden Materialien zu passivieren und damit den biologischen Bewuchs zu reduzieren, sollte das Bewuchsverhalten der zweiten Gruppe über die Umgebungstemperatur kontrolliert werden können. Die erste Gruppe wurde weiter in nichtfaulende Oberflächen für biomedizinische Anwendungen und auf der anderen Seite marine Anwendungen unterteilt. Für biomedizinische Anwendungen wurde dendritisches Polyglycerol (dPG), methoxyliertes dPG (dPG(OMe)), und lineares methoxyliertes Polyglycerol (lPG(OMe)) als Beschichtung von Glasfläschchen evaluiert, die als Primärverpackungsmaterial für hochkonzentrierte therapeutisch relevante Proteinlösungen dienen. Um eine praktische Aufbringung der Beschichtungen bei niedrigen Temperaturen und ohne Verwendung von Schutzgas zu ermöglichen, wurde die zuvor publizierte Beschichtungsprozedur entsprechend geändert. Alle beschichteten Glassubstrate zeigten eine signifikant reduzierte Proteinadsorption im Vergleich zu unmodifizierten Glasfläschchen, sogar nach Sterilisation mittels Hitze oder unter Druck. Besonders dPG basierte Beschichtungen zeigten eine ausgezeichnete Stabilität gegenüber den getesteten und industriell relevanten Sterilisationsmethoden. Weitere Stabilitätstests dieser über Siloxanbindungen immobilsierten dPG-basierten Beschichtung legten deren Anfälligkeit gegenüber hydrolytischer Spaltung unter basischen Bedingungen offen, aber auch deren Langzeitstabilität von mindestens drei Monaten unter lagerungsrelevanten Bedingungen.

dPG wurde außerdem als Beschichtung für Glas-Behälter für niedrigkonzentrierte Proteinlösungen getestet, wo der prozentuale Verlust aufgrund unspezifischer Adsorption deutlich höher war als bei hohen Konzentrationen, die gewöhnlich bei der Lagerung von Proteinlösungen verwendet werden. Durch das Aufbringen dPG(OMe) basierter Beschichtungen auf Probenfläschchen, die in einem SPR-basierten Immunassay als Behälter für Proteinlösungen verwendet wurden, konnte gezeigt werden, dass besonders bei niedrigen Proteinkonzentrationen eine dPG(OMe)-Beschichtung den prozentualen Proteinverlust aufgrund unspezifischer Adsorption deutlich reduzieren konnte. Hierfür wurde ein monoklonaler Antikörper (mAk) gegen ein auf der Oberfläche immobilsiertes und in der Alzheimer Krankheit auftretendes Peptid (Amyloid Beta 1-40) als Analyt verwendet. Der mAk wurde hierzu in unbeschichteten beziehungsweise dPG(OMe) beschichteten Probenfläschchen gelagert, bevor er in dem SPR basierten Immunassay verwendet wurde. Das deutlich reduzierte Signal für Analyt aus unbeschichteten Fläschchen im Vergleich zu Analyt aus beschichteten (23% Signalverlust bei der

niedrigsten Konzentration von 1 μg mL^{-1}) hat gezeigt, dass proteinresistente Beschichtungen essentiell sind für die Handhabung niedrigkonzentrierter Proteinlösungen in Proteinassays. Für diesen Zweck stellen die gesteten Beschichtungen eine exzellente Alternative gegenüber gewöhnlich verwendeten Passivierungsreagenzien wie Albumin dar.

Für den oben beschriebenen Immunassay wurde carboxymethyliertes dPG (CM-dPG) synthetisiert und als Immobilsierungsmatrix für Amyloid Beta 1-40 auf der Goldsensoroberfläche verwendet. Hierbei musste CM-dPG zwei Funktionen erfüllen: die Oberfläche resistent gegenüber unspezifischer Proteinadsorption machen, und zweitens die kovalente Immobilisierung von Peptiden ermöglichen. Adsorptionsexperimente haben gezeigt, dass das Einführen von Carboxymethylgruppen in dPG eine Verdopplung der unspezifischen Fibrinogenadsorption verursacht. Trotzdem, wurde die Fibrinogenadsorption gegenüber einer unbeschichteten Goldoberfläche immer noch um mehr als 99% reduziert, und gegenüber einem CM-Dextran-beschichteten Sensor um einen Faktor sechs. Um zu zeigen, dass CM-dPG zur Immobilsierung von Biomolekülen geeignet ist, wurde Amyloid Beta 1-40 kovalent an die Matrix gebunden und erfolgreich in dem oben beschriebenen Immunassay verwendet um die Wechselwirkung mit dem entsprechenden mAk (Anti-Aβ1-40) bei verschiedenen Konzentrationen zu messen.

Außerdem wurde ein lineares glycerol-basiertes Copolymer auf seine Eignung getestet verschiedene marine Organismen am Anhaften an Glas zu hindern. Um die Aufbringung dieser Beschichtung auf großen Substraten zu vereinfachen, wurde die Beschichtung über ein Sprühverfahren aufgebracht, wohingegen alle oben gennaten Beschichtungen durch Eintauchen des Substrats aufgebracht wurden. Es wurde gezeigt, dass die Fibrinogenadsorption sowie die Anhaftung mariner Bakterien (< 1%), Grünalgen (< 3%), und Miesmuscheln (< 15%) im Vergleich zu unbeschichtetem Glas deutlich reduziert werden konnten. Zusätzlich wurde gezeigt, dass die entwickelte Beschichtung bezüglich der Miesmuscheladhäsion besser abschnitt als Polytetrafluoroethylen (PTFE). Die Beständigkeit der Beschichtung ist im Meerwasser auf ein paar Wochen limitiert.

Desweiteren wurde eine responsive polymere Beschichtung beschrieben, die es ermöglichte die Zelladhäsion über die Umgebungstemperatur zu kontrollieren. Hierfür wurde eine Reihe linearer glycerolbasierter thermoresponsiver Copolymere hergestellt, die aus zwei verschiedenen Comonomeren aufgebaut waren und eine terminale Thiolgruppe aufwiesen. Während die Comonomere dazu dienten über deren molares Verhältnis die Schalttemperatur einzustellen, ermöglichte die terminale Thiolgruppe eine Immobilsierung auf Goldsubstraten. Um die

Schalttemperaturen der immobiliserten Polymere messen zu können, wurden die hydrodynamische Radien entsprechend beschichteter Goldnanopartikel als Funktion der Zeit bestimmt. Diese Ergebnisse haben gezeigt, dass die Schalttemperatur der Copolymere abnimmt, wenn sie auf einer Oberfläche immobilisiert werden. Es wurde ebenfalls gezeigt, dass alle getesteten Beschichtungen unterhalb der jeweiligen Schalttemperature hervorragende proteinresistente Eigenschaften aufweisen und oberhalb der jeweiligen Schalttemperatur eine stark proteinanziehende Wirkung haben. Schlussendlich wurde eine thermoresponsive Beschichtung als Substrat in der Zellkultur verwendet. Konfluente Zellschichten, die auf eben diesen thermoresponsiven Substraten kultiviert und anschließend auf collagenbeschichtete Kulturschalen transferriert wurden, zeigten eine exzellente Zelllebensfähigkeit.

7 References

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8 Publications, patents, and conference contributions

Publications

1. T. Becherer, M. V. Nascimento, J. Sindram, P.-L. M. Noeske, Q. Wei, I. Grunwald, R. Haag, *Fast and Easily Applicable Glycerol Based Spray-coating*, Prog. Org. Coat. **2015**, *87*, 146-154.

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Patents

1. R. Haag, M. Weinhart, I. Grunwald, Q. Wei, T. Becherer, PCT/EP 2014/069091, EP 13184328.6, *Multischicht-Architektur als Antifouling-Beschichtung für vielfältige Substrate* (*Hierarchical polymer multilayer architecture as bioinert coating*).

2. R. Haag, M. Weinhart, T. Becherer, U. Schedler, S. Küchler, K. Höger, W. Frieß, DE 102011 081260A1, EP 2744762A1, WO 2013026795A1, *Verfahren zur Herstellung von beschichteten Glasoberflächen und Glasgefäßen zur Handhabung von Biomolekülen oder biologischen Materiealien sowie die entsprechenden Glasgefäße* (*Method for producing coated glass surfaces*).

Oral presentations

1. Berliner Biacore Anwendertag, Robert Koch Institut, Berlin, 18 June 2009, *Proteinresistente Oberflächen auf Basis linearer und hochverzweigter Polyglycerine*, Tobias Becherer, Rainer Haag.

2. German Biacore and MicroCal User Meeting, EMBL Heidelberg, 27 September 2011, *Glycerol Based Polymers for Bioinert and Switchable Surfaces*, Tobias Becherer, Rainer Haag.