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Glycerol-based Polymers for Non-fouling Surfaces and Selectin Inhibition

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Contents

1 Introduction

1.1 General Aspects

In life sciences, there is a strong demand for the development of biocompatible materials including, e.g., drug and gene delivery systems for therapeutics, coating materials for implants and medical devices, as well as safe, new biomaterials with adjustable physical and chemical properties.[1, 2] In this respect, the question for a valid definition of a biomaterial or the term biocompatibility in general is essential. Due to the multifaceted applications of biomaterials, a universal answer is almost impossible. In fact, these definitions must always be adjusted to the respective application and desired function of the material. In a recent leading opinion paper the controversy of current definitions for biomaterials was addressed. Therein, a definition which was adapted to the rapidly growing amount of vastly different materials frequently applied nowadays in the biomedical field was suggested: *A biomaterial is a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine*.[1]

In addition to the desired physical and chemical properties such as mechanical strength or biodegradability, a biomaterial has to perform safely when in contact with living systems. Quoting D.F. Williams: *Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy.*[3] Despite being nontoxic, non-immunogenic, as well as physically and chemically inert, common biomaterials, however, often trigger a wide variety of adverse host responses.[4] This includes non-specific protein adsorption, cell adhesion, leukocyte and platelet activation, complement activation, and others as part of the foreign body reaction which often result in fibrosis,[5-8] inflammation,[9-14] thrombosis,[15, 16] and infection.[17-19] In fact, compromises are often made when pursuing certain functions and properties of implants or biotechnological devices depending on the respective application, while a reasonable, inevitable generic host response has to be accepted. Hence, with the two given paradigms in mind, a biomaterial as the substrate of an implant or a biomedical

device should further be specified as biocompatible with respect to its function and generic host response.

With the clinical experience of now more than 50 years of operated medical implantations a more in-depth understanding of the foreign body reaction has emerged.[20] In addition to tissue-engineering, bio- and nanotechnology which include modern non-invasive diagnostics as well as gene and stem cell therapy have been developed. As a result, the focus in biomaterials sciences has shifted from the perspective of materials science, which was based on metals, alloys, ceramics, and polymers in the beginning towards hybrid and "intelligent" materials often used in combination with biological entities nowadays.[21] Thereby, the chemical and biological inertness of the materials was initially considered as the ultimate goal because materials for classical long-term implantations were mostly pursued. Over time, however, functional, bioactive materials with specific responses to the living system have also entered the field.[22, 23] An example is the drug-eluting arterial stent which regulates the inflammatory and cell proliferating response of wound healing after implantation via continuous delivery of an anti-inflammatory and anti-proliferative drug.[24] Thereby the therapeutic is commonly located in the polymer coated metal stent surface and prevents restenosis.[25]

In addition to the common wound healing and inflammatory response to a biomaterial after implantation a persistent chronic inflammation often emerges which indicates a bacterial infection at the site of the biomaterial. Alternatively, endotoxins on the implant surface which originate from the cell membrane of dead bacteria act as antigens. They also activate the innate immune response and lead to adverse acute or chronic inflammation after implantation.[26] Such persistent inflammations lead to necrosis of the surrounding tissue or rejection of the implant and often require its removal as the only successful therapy.

Besides persistent chronic inflammation, thrombus formation at sites of medical devices in contact with blood such as intravenous catheters is often observed. A common clinical praxis therefore is to physically coat transient invasive intravascular catheters with heparin in order to lower the risk for thrombus formation as well as extensive loss of injected drugs at the otherwise hydrophobic catheter vessel wall due to non-specific adsorption.[27, 28] Heparin is a sulfated, branched polysaccharide isolated from mucosal tissue of porcine intestine or bovine lung. Systemically applied heparin has long been

known for its strong anti-coagulant effect and moderate anti-inflammatory properties.[29, 30] A non-animal derived, fully synthetic polymeric heparin analogue, however, would be desirable.

The first part of this thesis focuses on surface modifications for bioinert biomaterials, while the second part deals with the synthesis and properties (physical and biological) of polyanionic macromolecules as novel heparin analogues.

1.2 Bioinert surfaces

1.2.1 Biocompatibility

Many state-of-the-art materials for long-term implantable devices generally referred to as biomaterials such as polyurethane, silicones or polyester textiles are safe as a bulk material. These materials, however, do not show a bioinert surface since proteins or other bioentities readily adsorb on their surface via non-specific, i.e. hydrophobic or electrostatic interactions.[31, 32] Such non-specific protein adsorptions on implants or medical devices occur within the first few minutes after implantation and are considered as the first step of the foreign body reaction which is a part of the common wound healing process after implantation (Fig. 1).[4, 20] Thereafter, leukocytes (mainly neutrophiles and macrophages) recognize the biomaterial, become activated, and adhere to it within the time frame of one hour to one day. Since macrophages are too small to eliminate the biomaterial via phagocytosis, they fuse to form polynucleated foreign body giant cells within the first five days after implantation to better engulf the foreign body. These giant cells release cytokines which are small pro-inflammatory protein signalling factors that recruit further leukocytes and fibroblasts to direct the inflammatory and wound healing response to the site of the foreign body.[33] Since the giant cells are still not able to eliminate the biomaterial they release toxic mediators of degradation such as acid, reactive oxygen species (ROS) or degradative enzymes in a process termed frustrated phagocytosis.[34, 35] These mediators remain in particular in the shielded, privileged microenvironment between the giant cell membrane and the biomaterials' surface where they can partly oxidize and degrade the biomaterial in the long-term. The recruited fibroblasts synthesize collagen which surrounds the biomaterial and encapsulates it in an acellular bag within three weeks after implantation. If no chronic inflammation develops from a bacterial infection and no other adverse

immune response persists, the foreign material will become incorporated into the surrounding tissue within the final step of wound healing including new tissue formation and angiogenesis. However, complications are often observed after implantation of the biomaterial which can lead to rejection, i.e. the implant is not incorporated into the surrounding tissue but extensively encapsulated by collagen. Such fibrosis results in the improper function of the device. If an inflammatory response persists the biomaterial has to be removed by surgery.

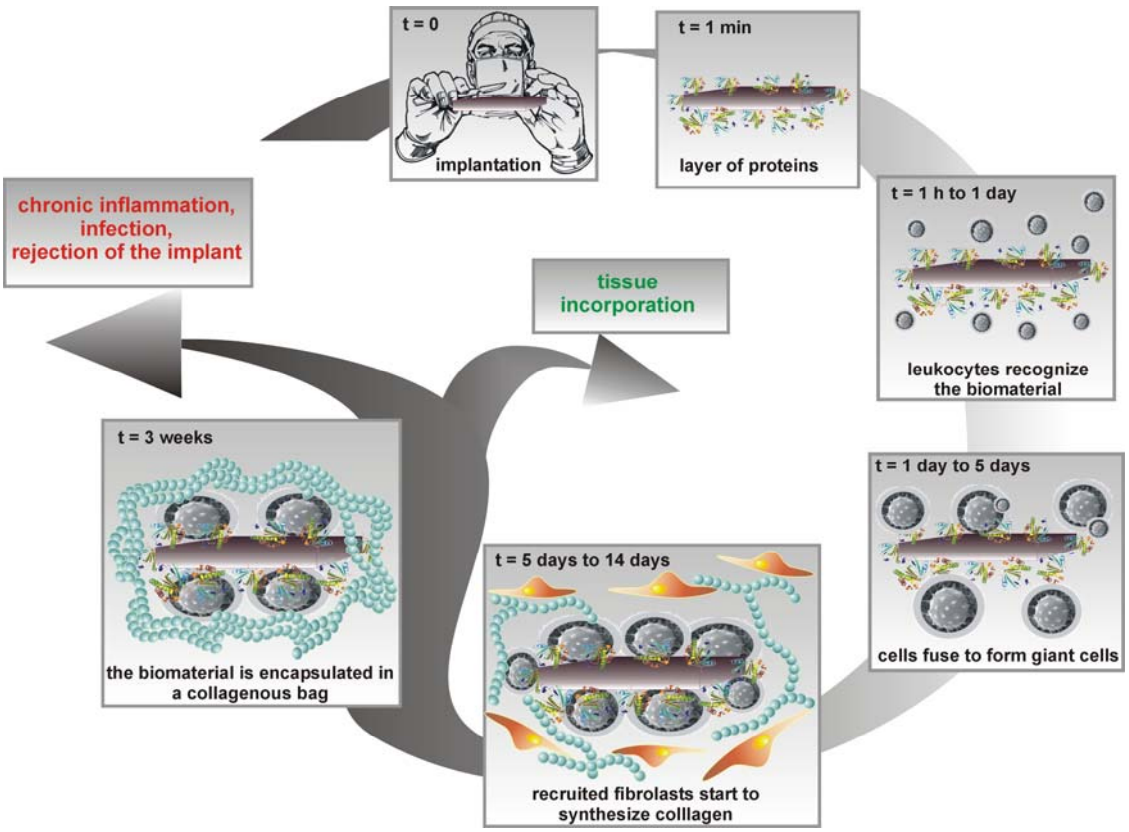


Figure 1. Illustration of the foreign body reaction in the course of the wound healing and inflammatory response to an implanted biomaterial.[36]

In addition to moderate complement activation (immune response) blood contacting biomaterials such as intravascular catheters or vascular stents have to be haemocompatible.[37] They should not induce blood coagulation or platelet activation,[38] and therefore should be protein resistant because adsorbed plasma proteins mediate thrombus formation. Adsorbed fibrinogen in particular promotes thrombosis since it becomes converted to fibrin by thrombin within the coagulation cascade and also mediates platelet activation.[39, 40] Unfortunately, high amounts of fibrinogen are always found within the adsorbed protein layer from whole blood or plasma.

It was further demonstrated that adsorbed fibrinogen on implanted biomaterials, in particular on hydrophobic surfaces, undergoes a conformational change. Thereby the binding strength to the biomaterial is enhanced via such denaturation which makes the adsorption irreversible.[41, 42] This conformational change was found to be critical since it leads to the exposure of two, usually (in case of soluble fibrinogen) hidden epitopes similar to those observed in the thrombin mediated conversion of fibrinogen to fibrin.[43] It is speculated that phagocytes may therefore recognize adsorbed fibrinogen as "fibrin" via a distinct integrin (Mac-1). As a result they attach to the biomaterial, become activated, and initiate an inflammatory as well as a wound healing response similar to the ones initiated by fibrin clot formation.[43, 44]

This emphasizes the need for bioinert and in particular protein resistant materials. Non-specific protein adsorption is the first event that occurs when a biomaterial is exposed to biofluids and all following events are mainly mediated by this adsorbed layer.[31, 45-47] This also explains why almost all materials used for implants including metals, ceramics, and polymers with soft or hard material properties meet the same fate in vivo in the course of the foreign body reaction. Therefore, protein resistant surfaces seem to offer a universal approach to enhance biocompatibility at least as far as blood contacting devices such as catheters, biosensors, and bioanalytical in vitro devices are concerned.[48-50] Since non-specific protein adsorption has a severe impact on biocompatibility, fundamental aspects of protein adsorption will be summarized in the following part.[31]

First of all, adsorption is favored or prevented by a number of enthalphic and entropic changes in the substrate-water-protein system. This includes dehydration of protein and surface, redistribution of charges at the interface, and conformational changes of the protein.[51] In general, proteins adsorb on surfaces mainly due to hydrophobic, van der Waals, and electrostatic interactions. It is often observed that hydrophobic surfaces adsorb more proteins than hydrophilic surfaces, but this also strongly depends on the nature of the protein (hydrophobicity, charge, conformational stability). Denaturation of the adsorbed protein is more likely on hydrophobic surfaces to increase contact sites which makes the adsorption irreversible. In contrast, the reduced protein adsorption on hydrophilic surfaces may be attributed to enthalpic effects. Hydrophilic surfaces as well as proteins are well hydrated in an aqueous environment. Therefore, interaction of the protein with the surface requires at least partial dehydration of the contact sites which

resembles an enthalpic barrier.[52] Another effect that impacts protein adsorption is the ionic strength and the pH of the medium. With increasing ionic strength the charges of the protein are more effectively shielded and become neutralized at a pH equal to the isoelectric point of the protein and therefore decrease electrostatic interactions with the surface. According to the Hofmeister series (see Chapter 1.31) of ions kosmotropes, small hydrated ions with high charge density such as SO_4^{2-}, usually stabilize proteins whereas chaotropes, large less hydrated ions with low charge density such as SCN^-, destabilize proteins at high salt concentrations.[53, 54] Kosmotropes increase the surface tension of water and therefore favour hyrophobic interactions of proteins thus resulting in salt-incuced precipitation of proteins, whereas chaotropes directly interact with the peptide sequences resulting in salt-induced increased solubility and denaturation of proteins.[55]

In general, charge neutral (including zwitterionic) surfaces adsorb less proteins than charged surfaces do.[51] In addition, surface topography (roughness and texture) influences protein adsorption. Smooth and homogenous surfaces show less adsorption than rough (on the nanometer scale) and porous surfaces.[56, 57] The latter have a larger surface area and therefore offer more potential contact sites with the protein while a concomitant change in the geometrical arrangement was observed for adsorbed fibrinogen. An exception to this are surfaces with a lotus leaf-like topography (on the micrometer and nanometer scale) as demonstrated with polyurethane(PU)/pluronic® blends which showed reduced fibrinogen adsorption (> 94% compared to smooth, bare PU) and no cell adhesion.[58] These lotus leaf-like surfaces show three dimensional elevations of 5-10 µm diameter at a distance of 10-30 µm and a superimposed fine structure with dimensions of 10 nm-5 µm.[59, 60] The non-adhesive, self-cleaning nature of such surfaces is attributed to limited contact sites with particles or proteins.

Furthermore, protein concentration and in particular the composition of the protein mixture have a crucial impact on the amount as well as on the composition of the adsorbed protein layer. Leo Vroman discovered that protein adsorption from mixtures such as plasma is a rather dynamic process.[61] Small and readily available proteins which are present in high concentrations such as albumin (60% of plasma proteins) adsorb first. In the case of reversible adsorption they are replaced over time by proteins with higher affinity for the surface which are present in lower concentrations such as

fibrinogen (Figure 2A). This effect is more pronounced on hydrophilic surfaces on which proteins are commonly less tightly adsorbed.

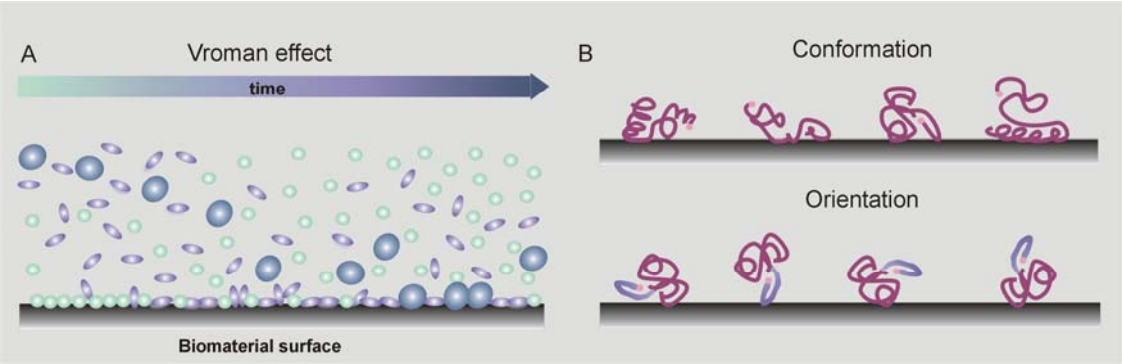


Figure 2. A) Illustration of the Vroman effect. Proteins which are present in high concentrations in the medium adsorb first on the surface and get replaced over time by proteins with lower abundance in the medium but higher affinity to the surface. B) Illustration of orientation and conformational changes of adsorbed proteins.

Once adsorbed on the surface proteins may undergo conformational changes in order to enhance contact sites with the surface, as mentioned above.[62] In addition, the loss of secondary structure of a protein, e.g., the transition of a α-helix to a random coil, increases chain flexibility and therefore entropy. This is the main driving force for protein adsorption on otherwise prohibitive hydrophilic or – with respect to the proteins charge – likely charged surfaces. It was further demonstrated that adsorbed proteins are not fully denaturated. For example, bovine serum albumin (BSA), a flexible, globular protein, could fully retain its configuration after desorption from hydrophilic surfaces but not from hydrophobic surfaces.[62] The relatively stiff protein lysozyme could regain its native configuration even after desorption from hydrophobic Teflon surfaces.[63] Such conformational changes might also be induced by the underlying topography of the surface and translate into the subsequent cellular host response to an implanted biomaterial surface. Similarly, proteins can adsorb with different orientations on the surface depending on the above-mentioned parameters. Thereby, they might lose their activity because the essential active epitopes would then be buried. Both conformational changes as well as orientation of the adsorbed protein are illustrated in Figure 2B. Due to the hydration of adsorbed as well as dissolved proteins an energetic barrier exists for protein-protein interactions. Therefore proteins tend to adsorb on surfaces in monolayers and do not adsorb non-specifically onto their own monolayers.[31]

Since the development of biomaterials with innate protein resistant properties is rather challenging, a more common method is the surface functionalization of already established biomaterials with a biocompatible or more specified a protein resistant coating. In the following section model surfaces for a fast and efficient evaluation of materials for biocompatible coatings are presented.

1.2.2 Self-assembled monolayers (SAMs) on gold and glass as effective model surfaces

Surface modification techniques for controlling the properties of a material's surface including enhanced biocompatibility are manifold and depend on the nature and characteristics of the surface substrate and on the molecules or polymers that are used for surface modification. Self-assembled monolayer (SAM) formation has been established as a straightforward approach to surface modification.[64] The SAMs offer well defined structures that can be systematically altered by the chemistry of the respective self-organizing molecules and the substrate surfaces. Furthermore, spatial patterning can be achieved via, e.g., microcontact printing[65] or photolithography.[66, 67] Self-assembly is a spontaneous process to build up ordered hierarchical structures and is often encountered in nature, e.g., lipid membrane formation. A SAM on a surface is defined as a spontaneously formed ordered layer of molecules with specific affinity of their headgroups to the surface. They can be generated from thiols on metal or semiconductor surfaces such as Au, Ag, Pd, Pt, Cu, Hg, CdSe, and ZnSe.[68, 69] In addition, oxidic substrates, e.g., Al_2O_3, TiO_2, and SiO_2 can be modified with SAMs from molecules comprising carboxylic acid and phosphonate headgroups or silane groups, respectively.[64] The far most studied systems are silyloxy tethered monolayers on glass or mica as well as thiol, disulfide or sulfide tethered monolayers on gold. Such monolayers can either be generated in liquid phase by immersing the substrate into a solution of the SAM forming molecules. Thereby, alkanethiol SAM formation is observed immediately within the first few seconds whereas over time (1-48 h) the SAM remodels to adapt an upright all-trans zig-zag conformation with highest possible lateral chain density. This in turn maximizes van-der-Waals interactions of the molecules as the secondary driving force besides the headgroup-surface interaction.[70] Alternatively, vapor deposition can be used to generate SAMs. SAMs on metals in general are more ordered than SAMs on glass since the underlying metal lattice dictates the position of

the anchoring headgroups. Moreover, the sulfur-gold binding is reversible and hence allows mobility of the SAM forming molecules on the surface for optimized packing. In case of a Au(111) surface and alkanethiols as the assembling molecules the thiol groups, which have a binding energy to Au of approximately 50 kcal/mol, adapt a distance of approximately 5 Å to their closest neighbours. In addition, the alkyl chain attached to the sulfur atom tilts by approximately 30° against the surface normal in order to maximize van-der-Waals interactions of the alkyl chains.[64] One methylene unit contributes approximately 1.5 kcal/mol. Due to this tilt angle the tailgroup of alkanethiols with an even number of methylene groups is aligned more parallel to the surface while the one of alkanethiols with an odd number of methylene groups is aligned perpendicularly to the surface.[71] This has direct implications on the surface properties of the SAM, e.g, the wettability or the reactivity of functional tailgroups. In contrast, silane based monolayers commonly tethered via trichlorosilyl, trimethoxysilyl, or triethoxysilyl groups show a significantly lower lateral chain density and less ordering than SAMs on gold.[72] The most striking difference to SAMs on gold is the partial crosslinking of the silyl SAM after attachment to the surface and hence no mobility of the SAM forming molecules on the surface after the initial attachment. This leads to a higher mechanical and thermal stability of the SAM but lower ordering. In addition, alkylsilanes are usually not tilted and arrange perpendicular to the surface (Figure 3).

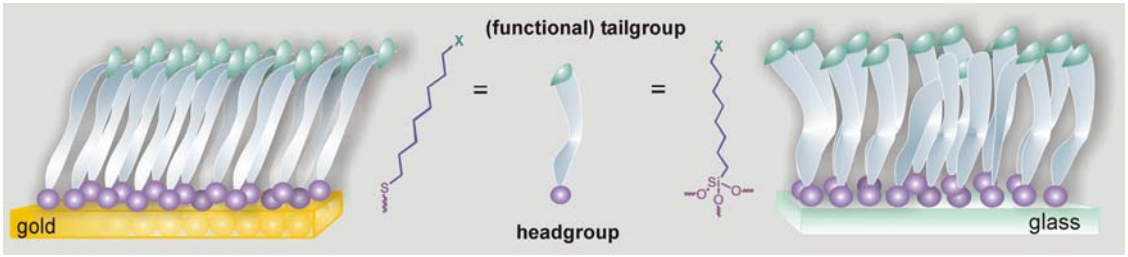


Figure 3. Illustration of a thiol-based SAM on gold (left) and a silyloxy-based SAM on glass (right).

Due to the sensitivity of trichlorosilanes and trialkyloxysilanes towards hydrolysis the absence of water during the surface immobilization step is critical. Hydrolysis results in the formation of an unreactive hydroxyl group for surface immobilization at the silyl tether. Such hydroxyl groups, however, are reactive towards polymerization with other non-hydrolyzed silyl groups and thus can lead to multilayer formation on the surface.

Therefore, silyloxy tethered monolayers are somewhat more challenging to produce than SAMs on gold but offer higher stability than the latter ones.[70]

Besides the relative straightforward modification of surfaces with SAMs they can be characterized by a variety of analytical techniques. A few examples are IR reflection absorption spectroscopy (IRRAS) and surface plasmon resonance (SPR) spectroscopy on gold surfaces, as well as X-ray photoelectron spectroscopy (XPS), ellipsometry,[73] quartz crystal microbalance (QCM),[74] microscopy including atomic force microscopy (AFM) and fluorescence microscopy, contact angle measurements, and time-of-flight-secondary ion mass spectrometry (ToF-SIMS)[75] on both gold and glass substrates, respectively.[76] All these analytical tools not only support a proper characterization of the bare SAM surface but also allow for the evaluation of biological interactions with these SAM surfaces.[77, 78] Therefore, SAM modified surfaces have been extensively used in the past twenty years to study protein-surface and cell-surface interactions.[79]

A powerful tool in this respect is SPR spectroscopy (for a description of the physical principal see Chapter 7.1) since it allows for the real-time investigation of the non-specific protein adsorption.[80] A typical sensorgram obtained from such measurements, which is a time-dependent plot of the response signal given in response units (RU), is displayed in Figure 4. Initially the SAM mofified gold surface is placed into a microfluidic cartridge of a SPR device and equilibrated by passing buffer solution such as PBS over the surface for a few minutes until a stable baseline is obtained. Subsequently a protein solution in buffer is injected and passed over the surface while the response signal increases due to adsorption of proteins on the surface. After 25 minutes the surface is rinsed again with PBS in order to remove loosely adsorbed proteins while the response signal decreases due to protein desorption until a stable baseline is obtained. The difference in response units (ΔRU) after protein injection and desorption and before protein injection then corresponds to the amount of truly adsorbed proteins. In Figure 4 two such curves for a hydrophobic hexadecane thiol (HDT) and a hydrophilic methoxypoly(ethylene glycol) (mPEG) SAM surface are shown with fibrinogen as the test protein. Obviously the hydrophobic HDT surface adsorbs much higher amounts of fibrinogen than the mPEG modified SAM surface. The latter is currently considered as benchmark for bioinert surfaces. Since SPR is a relative method the HDT surface is often used as a reference surface and the corresponding ΔRU value is set to 100% protein adsorption equaling a monolayer of adsorbed

proteins. The determined ΔRU form the sample surfaces like the mPEG surface can then be transformed to % protein adsorption (PA) with respect to a HDT reference surface according to equation (**1**) for comparison with other surfaces.

$$\%PA = \frac{\Delta RU_{sample}}{\Delta RU_{HDT}} \cdot 100\% \qquad (\mathbf{1})$$

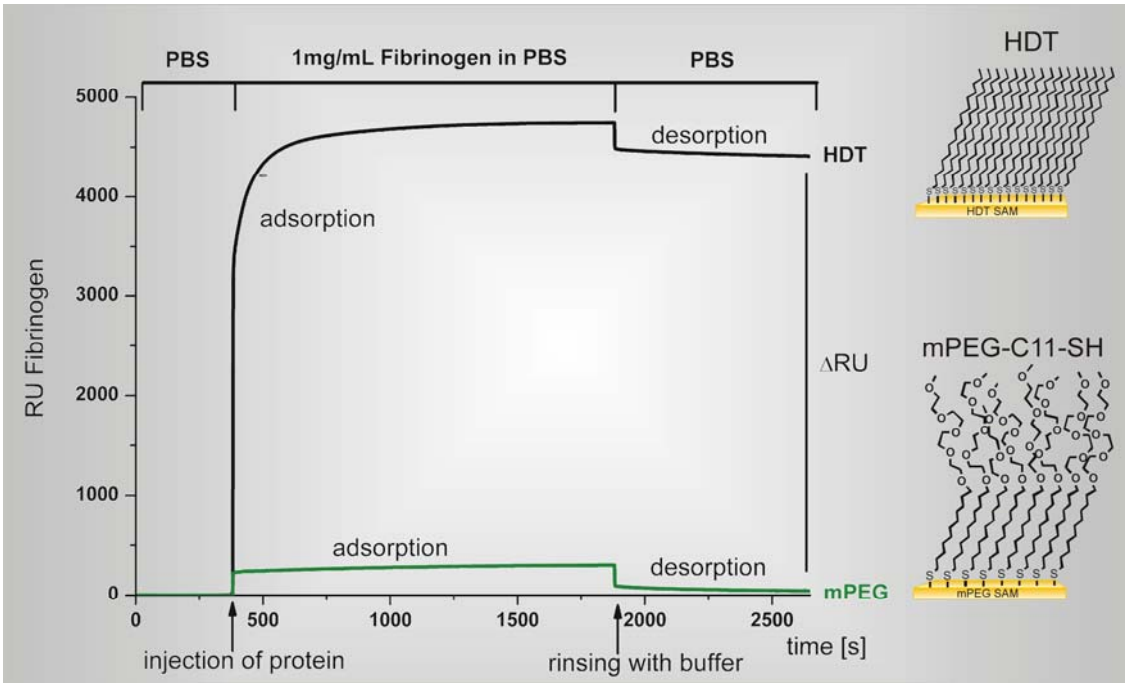


Figure 4. Typical SPR-curves obtained from the evaluation of the non-specific protein adsorption on model SAM surfaces. Hexadecane thiol (HDT) on gold is used as a hydrophobic reference surface (ΔRU_{HDT} = 100% protein adsorption) and mPEG-C11-SH is considered as a benchmark for protein resistant surfaces.

It has long been known that mPEG modified surfaces show enhanced biocompatibility with respect to protein adsorption, cell adhesion, and complement activation. Meanwhile a variety of other hydrophilic polymers has been identified to also enhance biocompatibility when tethered on surfaces (see Chapter 1.2.3). When functional groups on top of an undecylthiol based SAM on gold were surveyed for fibrinogen adsorption, however, it turned out that hydrophilic hydroxyl groups adsorbed large quantities.[81] In comparison with amine, methyl or carboxylic acid groups on top of such a SAM layer fibrinogen adsorption increased in the order OH < COOH < CH_3 < NH_2.[82] This can partially be explained by the inherent physical properties of fibrinogen. Fibrinogen is a large (340 kDA) blood plasma protein with a pI ~ 5.5 and therefore negatively charged under physiological conditions.[83] This causes an increased electrostatic interaction with

partial positively charged amine functionalized surfaces and an electrostatic repulsion in the case of partially negatively charged carboxylic acid functionalized surfaces. Despite the fact that the overall charge of fibrinogen is negative there are patches of negative as well as of positive charges on its surface, whereby the latter ones contribute to the interaction with negatively charged (e.g. COOH) surfaces. The interaction of fibrinogen with methyl terminated SAMs is mainly hydrophobic in nature which leads to enhanced conformational changes and in turn makes the adsorption irreversible. This can also be seen from the desorption part of the SPR sensorgram of hydrophobic HDT SAMs (cf. Fig. 4) which quickly after starting the PBS rinsing step yields a stable baseline without an extended decaying SPR signal and therefore indicates largely irreversibly adsorbed proteins.[82] Fibrinogen adsorption followed by a conformational change is pivotal for thrombin mediated fibrin formation and platelet activation within the cascade of thrombus formation.[83, 84] Thrombin interaction with preadsorbed fibrinogen on hydroxyl, amine, carboxylic acid, and methyl terminated SAMs studied via SPR revealed that fibrin formation was highest on methyl and amine functionalized SAMs while hydroxyl terminated SAMs showed moderate fibrin formation and carboxylic acid functionalized SAMs did not show any fibrin formation although non-specific fibrinogen adsorption was high.[82] These results indicate the amount of conformational changes which occurred during fibrinogen adsorption on OH, NH_2, and CH_3 terminated SAM surfaces. In the case of a carboxylic acid functionalized SAM surface the authors suggested two possible explanations. Either adsorbed fibrinogen does not undergo enough conformational changes to allow specific thrombin binding for the enzymatic fibrinopeptide cleavage for converting fibrinogen to fibrin or the negatively charged surface competes with adsorbed fibrinogen for the binding of thrombin. Although thrombin is predominately uncharged it was demonstrated that it non-specifically adsorbed on negatively charged surfaces and that the specific binding to fibrinogen is mediated by cationic clusters on the surface of thrombin.[85]

SAM surfaces have also been applied to evaluate the role of certain chemical functional groups for their effect on biocompatibility in terms of complement activation or inflammatory response.[86] Biomaterial related complement activation is thought to be accomplished via the alternative pathway. From numerous studies it was suggested that hydroxyl and amine nucleophiles activate the complement system while negatively charged sulfate or carboxylate groups do not activate the complement system, but this hypothesis does not hold true in every case.[86-88]

1.2.3 Survey of protein resistant surfaces and suggested mechanistical hypotheses

Since proteins typically adsorb as monolayers on surfaces, a commonly applied strategy to reduce the non-specific interaction of proteins with surfaces is the formation of a physisorbed albumin monolayer by dip coating.[31] Such preadsorbed protein monolayers, however, are not stable and hence show only a limited short-term effect. Synthetic surface modification by a covalent attachment of appropriate molecules is a more promising way to render surfaces protein resistant and non-fouling for long-term applications.[89] In the pursuit of non-fouling surfaces Whitesides and coworkers performed a broad screening study on the structure-property relationship of more than fifty molecules via reactive SAM formation and SPR analysis.[90] From their results the authors derived four empiric rules: a protein-resistant compound should be i) overall neutral, ii) hydrophilic, iii) have hydrogen bond acceptors and iv) no hydrogen bond donors. Many compounds which have been identified nowadays to have protein resistant properties fulfill these criteria yet exceptions to these empirical rules also exist. Besides a few low molecular weight compounds mainly polymers have been found to show non-fouling properties. The latter ones are much more promising for application of, e.g., surface coating of metals or other polymers since they cover a larger surface area per molecule. Despite now more than 20 years of intense experimental and theoretical work on this topic the molecular mechanism of protein resistance is still not fully understood.[91] Within this section only polymers will be considered and the current mechanistical hypothesis for their protein-resistant properties will be discussed.

The most frequently applied coating material for the generation of bioinert surfaces is the linear hydrophilic polyether poly(ethylene glycol) (PEG) also termed poly(ethylene oxide) (PEO) as well as its lower molecular weight analogue oligo(ethylene glycol) (OEG) (Fig. 5).[50, 92, 93] Thereby the terminal group can be either a free hydroxyl group or a methoxyether group which is then usually indicated by the term mPEG. Disadvantages of PEG such as oxidative degradation in the presence oxygen and transition metals and related complement activation have prompted efforts to identify alternatives.[94] Thereby a variety of other hydrophilic, synthetic, linear, and neutral polymers have been identified to have protein resistant properties when tethered on surfaces such as triglycerol with either free hydroxyl (TG(OH)) or methoxy side chains (TG(OMe),[95] (poly(2-methyl- or ethyl-2-oxazoline) (PMeOx, PEtOx),[96-98] poly(vinyl

pyrrolidone) (PVP),[99, 100] poly(2-hydroxyethyl methacrylate) (PHEMA),[101, 102] and certain peptido-mimetics[103, 104] (Fig. 5). The most common explanation for the observed protein resistant properties of such surfaces is steric repulsion of the tethered polymer chains. The polymers displayed in Figure 5, as well as a variety of polysaccharides which have also been identified to show protein resistant properties,[105, 106] have a highly flexible structure in common which adopts a random coil conformation in solution. When a protein approaches the surface and adsorbs on the interface, the configurational freedom of the polymer chains becomes reduced which in turn leads to an entropical penalty. Furthermore, the hydrophilic polymers at the interface as well as proteins are well hydrated in aqueous solution. Adsorption of the protein at the solid-liquid interface would require at least partial dehydration of the protein and the tethered polymer chains in order to allow for hydrogen bonding.

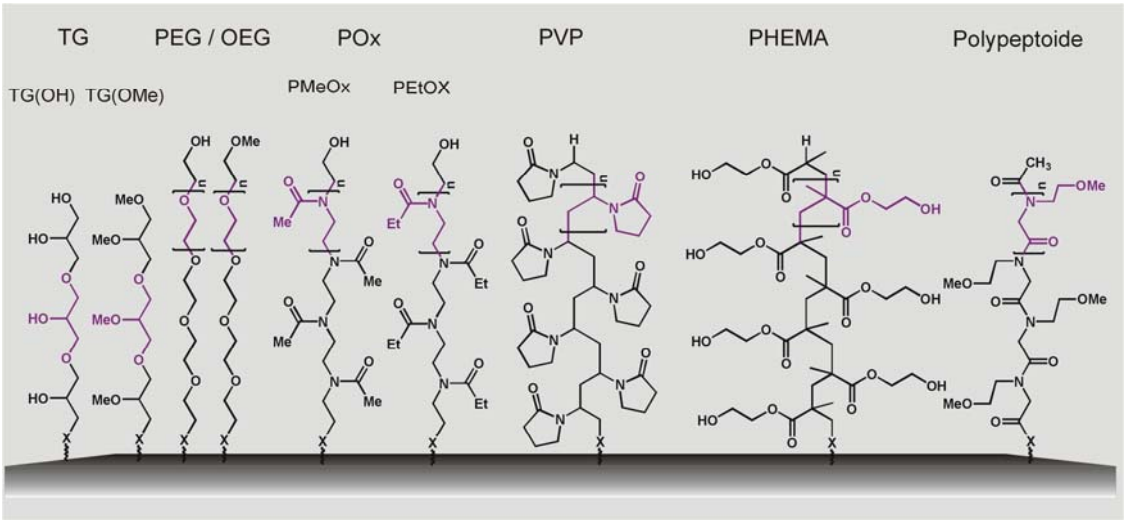


Figure 5. Structure of linear, synthetic, hydrophilic, and neutral polymers with protein resistant properties. Most of them are not capable to form strong secondary inter-chain interactions (e.g. hydrogen-bonding).

For the most intensively studied PEG/OEG system protein resistance was found to be dependent on the degree of polymerization as well as on the packing density of the molecules on the surface.[107-110] At low lateral chain densities protein adsorption was observed which is attributed to an insufficient shielding of the underlying surface. Also at very high packing densities protein adsorption was observed. The reasons for this are not entirely clear but it was suggested that the reduced chain flexibility along with reduced hydration of the PEG molecules are the major determinants.[108] However, such high lateral chain densities are just accessible via SAM formation on metal substrates

while on more applied polymeric substrates moderate grafting densities are usually achieved at best. Moderate grafting densities were shown to be in the optimal range for protein resistance while in general marginally lower grafting density can be compensated by longer PEG chains.[108]

Mechanistically steric repulsion and chain flexibility cannot fully account for the observed protein resistance of tethered OEG chains on gold surfaces.[110] This is particularly true because OEGs with only two repeating units already revealed as good protein resistance as OEGs with six repeating units as long as the chain density did not exceed a certain threshold for the latter one.[107, 111] In the case of OEGs protein resistance is attributed to the internal and external hyrophilicity of the SAM. The lateral chain density dictates the configuration of the tethered OEG chains which in turn determines the degree of hydration of the OEG chains. For SAMs on silver the atom lattice enforces a higher chain density than on gold substrates.[112] Methoxy terminated triethylenglycol SAMs on silver substrates are not protein resistant. The OEG chains of such a SAM were found to be in an all-trans configuration with reduced hydration of the OEG chains. In contrast, the same SAM on gold substrates revealed a helical or amorphous configuration that in turn allows for a higher degree of internal hydration and results in protein resistance.[113]

In addition to linear hydrophilic polyethers polymers with branched architecture were also found to be highly protein resistant. Among them were SAMs of dendritic polyglycerol,[114] star shaped PEG based polymers,[115, 116] and OEG bearing bottle brushes[48] (Fig. 6). Dendritic polyglycerol (dPG) is a highly flexible polyether which can be synthesized via a one-step anionic multibranching ring-opening polymerization of glycidol on a polyol initiator.[117, 118] This polymer was shown to be highly biocompatible with respect to cytotoxicity in vitro, acute toxicity as demonstrated via a mice model in vivo, and haemocompatibility including no complement and no platelet activation.[119] Protein resistance of dPG SAMs on gold was shown to be comparable or better than SAMs of PEG of similar molecular weight.[114, 120] Star shaped PEG (StarPEG) is a multiarmed branched polymer with a focal branching point. Depending on the multiplicity of this focal branching point the amount of PEG arms on the molecule can be adjusted, while the PEG chains are attached either via a graft-polymerization of ethylene oxide from a polyol initiator as the focal branching point or a grafting to approach with preformed, reactive PEG chains.[121] StarPEGs with a

relatively high number of PEG arms, high molecular weight PEG, and a rigid focal point revealed worse protein resistance than tethered single PEG chains.[122] This was attributed to a heterogeneous monolayer formation which leads to small non-shielded surface areas which are easily accessible for small proteins. Möller and coworker improved such StarPEG systems and designed cross-linked films in the range of 3-50 nm thickness which had better protein resistance than single tethered PEG chains.[123, 124] Oligo(ethylene glycol) methacrylate (OEGMA) bearing bottle brushes are commonly synthesized via surface initiated polymerizations (SIP) such as atom transfer radical polymerization (ATRP) on gold or silicon surfaces.[125, 126] This allows for a precise control over the degree of polymerization due to the "living" character of the polymerization mechanism. The graft density can be adjusted via SAM formation of the ATRP-initiator previous to the actual graft-polymerization. The resulting protein resistance of such surfaces is dependent on the thickness as well as on the density of the tethered bottle-brushes.[127] Above a dry thickness of the coating of ≥ 10 nm excellent protein resistance from single protein solutions as well as from complex mixtures such as serum was observed.[48] This is remarkable since total serum resistance is not achieved by common PEG monolayers.

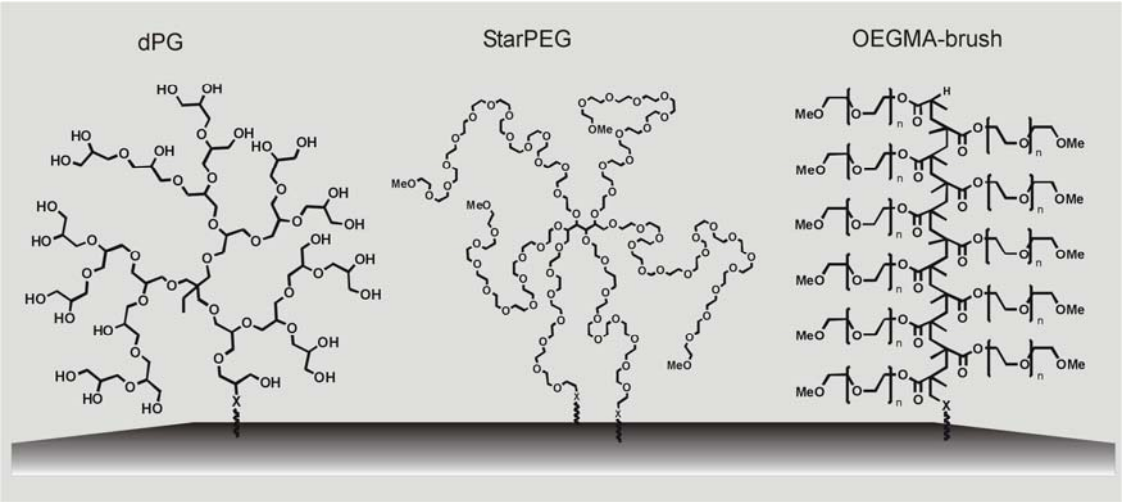


Figure 6. Structure of dendritic polyglycerol (dPG), star-shaped PEG (StarPEG), and oligo(ethylene glycol) methacrylate (OEGMA) based bottle-brush with protein resistant properties. All of them are highly flexible aliphatic polyethers with high ability for hydration and possible ion complexation from buffer.

All these branched polyether materials have in common that they are hydrophilic and highly flexible which leads to comparable protein resistance as observed with SAMs of PEG. The superior resistance, in particular with respect to complex protein mixtures, is

attributed to a more effective surface shielding by homogenously smooth monolayers of branched polymers as compared to their linear analogous. In addition, such polymers form SAMs which are more disordered in their interior structure than their linear analogues due to the branching and this in turn yields more space for internal hydration of the SAM.[48] A common feature of these branched architectures is the possibility for the introduction of specific ligands via their multiple functional groups in order to generate, e.g., biospecific surfaces with improved signal to noise ratio. In case of the given structures of StarPEG and the bottle brush in Figure 5, the methoxy terminus then has to be replaced by, e.g., a reactive hydroxyl terminus.

Another class of protein resistant polymers is made up from zwitterionic polymers. Zwitterionic phospholipids constitute the major component of biological membranes and are generally considered to be non-thrombogenic. Polymers based on the zwitterionic phosphocholine (PC) headgroup of such phospholipids are easily accessible via radical polymerization.[128, 129] Surfaces modified with polymers based on 2-methacryloyloxyethyl phosphorylcholine (PCMA) revealed reduced protein adsorption (Fig. 7).[130] The protein resistance was found to be dependent on the flexibility, i.e. the chain length of the alkyl spacer between the ionic groups and the grafting density of the brushes.[131] In addition, mixed low molecular weight SAMs on gold with alternating positively and negatively charged tailgroups at the interface in a 1:1 ratio revealed reduced protein adsorption similar to OEG SAMs.[132, 133] Based on these findings Jiang and coworkers developed zwitterionic sulfobetaine methacrylate (SBMA) and carboxybetaine methacrylate (CBMA) based brushes via ATRP on various surfaces.[134] These surfaces were shown to be highly resistant towards protein adsorption even from complex mixtures such as undiluted serum or plasma and performed better than surfaces modified with OEGMA-based brushes (Fig. 7).[135-138] The protein resistance of these zwitterionic brushes is attributed to steric repulsion of the flexible polymer brushes and their high degree of hydration. From model studies on PC SAMs it is known that the ratio of negative and positive charges has to be exactly 1:1 or the neutral character will be lost which results in enhanced electrostatic protein adsorption.[139] If zwitterionic monomers are used, fine tuned charge balance is less of a problem compared to zwitterionic brushes which are synthesized from statistical polymerization of cationic and anionic comonomers.[134] By means of PC model SAMs it was further demonstrated that the zwitterionic tailgroups have to arrange in a way that the dipole vectors of

adjacent tailgroups are aligned antiparallel in order to minimize the electrostatic energy and net dipolemoment of PC SAMs.[139] In this respect zwitterionic brushes easily adapt optimal configuration due to the flexibility of the brushes. However, at low pH the CBMA based polymer becomes partially protonated which in turn offsets charge neutrality. In general, zwitterionic polymers in solution show antipolyelectrolyte behaviour and expand at high ionic strength while the polymer chains collapse at low ionic strength conditions.[140] Hence, protein adsorption increases at low ionic strength, in particular, for bare sulfobetaine or PC SAMs, while CBMA based brushes can partially compensate this by their freely fluctuating sidechains.[138]

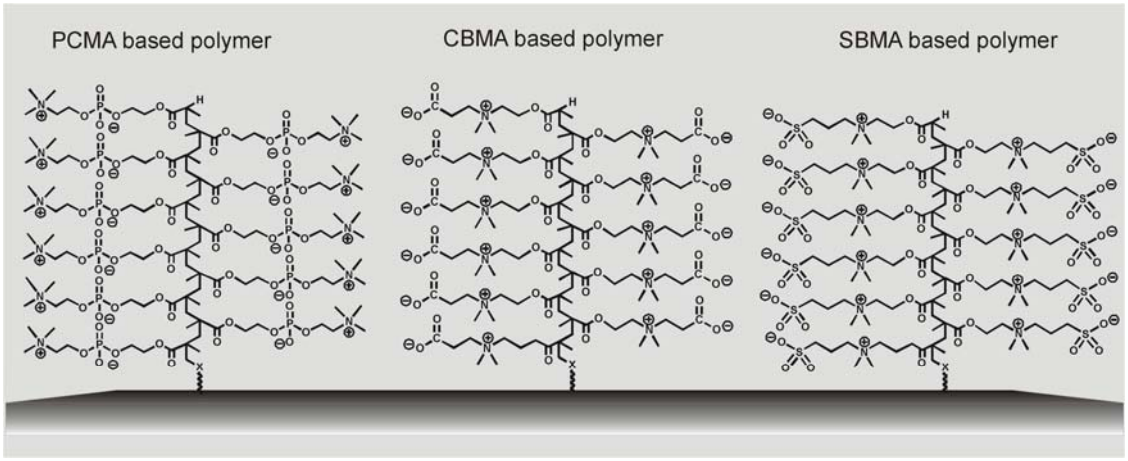


Figure 7. Structure of zwitterionic phosphocholine methacrylate (PCMA), carboxybetaine methacrylate (CBMA), and sulfobetaine methacrylate (SBMA) based brushes that resist the adsorption of proteins. All of them show a high degree of hydration and high ionic strength within the brushes due to confined counter ions under physiological ionic strength of the solution.

Another important feature of zwitterionic brushes is the presence of associated counter ions within the polymer brush at high ionic strength, i.e. under physiological conditions. It is speculated that these confined counter ions contribute to the observed protein resistance since they would have to be at least partially released from the brush into the bulk upon protein adsorption similar to hydrating water molecules.[141] This might also be true for polyether surfaces which are able to easily complex small metal cations from the buffered environment. In Figure 8 the different suggested hypotheses on the mechanism of protein resistance are summarized by means of the above classified protein resistant polymers. The concept of steric repulsion applies to all categories of protein resistant polymers since linear hydrophilic polymers as well as branched polyethers or zwitterionic bottle brush polymers are relatively flexible polymers which

would suffer from a loss in entropy when proteins adsorb on them. Moreover, all of them are well hydrated due to their intrinsic hydrophilicity which generates an enthalpic barrier for protein adsorption that requires at least partial dehydration of the brushes and the protein. Moreover, polyethers can complex cations from physiological media which, together with their confined counter ions, form an osmotic barriere towards protein adsorption. Similar to water molecules within the polymeric brush, ions would have to be at least partially released into the bulk and thereby cause an unfavourable osmotic gradient. For branched polyethers all these considerations apply while the branched architecture guarantees a more effective steric shielding of the surface in contrast to linear analogues. This might explain the enhanced protein resistance of oligoethylene glycol based bottle brushes as compared to linear polyethylene glycol in particular if more complex protein mixtures such as serum are considered. Zwitterionic bottle brushes combine all these effects, since they are highly flexible and well hydrated due to the charges in their zwitterionic side chains. Moreover they guarantee an effective steric shielding of the surface due to their branched architecture and at the same time generate an osmotic barrier towards protein adsorption due to confinded counter ions within their brushes at high ionic strength conditions.

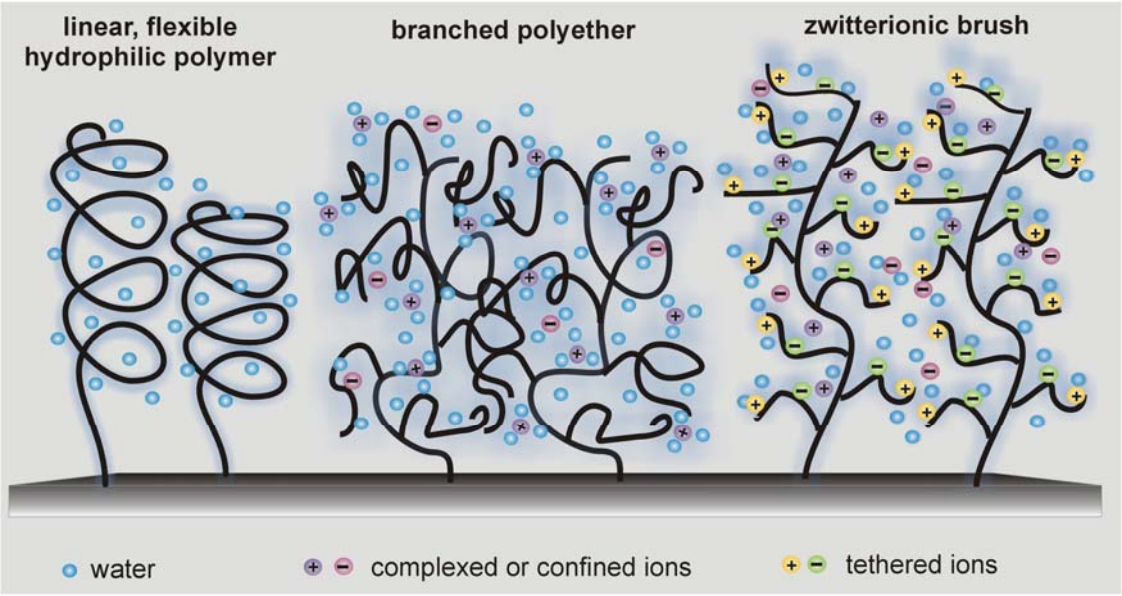


Figure 8. Illustration of suggested mechanistical hypotheses for protein resistant surfaces.

Although the mechanism of protein resistance is complex in nature and might involve several effects at the same time, a general guideline for the design of protein resistant surfaces can be concluded from the state-of-the-art. Thus, the tethered polymer chains

should be of sufficient length to be flexible, well hydrated, and should have an optimal packing density in order to effectively shield the surface but at the same time allow chain mobility. Furthermore, the surface tethered chains should bear groups that are readily accessible for hydrogen bonding to water molecules but not accessible to hydrogen bondable groups of the protein.[142] Furthermore, a high local ionic strength within the brush layer seems to be advantageous.

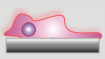
1.2.4 Protein mediated cell adhesion on surfaces

Cell adhesion on the surface of biomaterials and biomedical devices is commonly observed during the host response towards an implanted material or in vitro in the presence of cells. Many cell types require attachment to a surface in order to survive, while cell growth, proliferation, and phenotype are determined, in part, by the adapted morphology of the cell. It is generally believed that eukaryotic as well as prokaryotic cell adhesion is a mainly protein mediated process, but the detailed molecular mechanism is still under investigation.[17, 45]

Controlled eukaryotic cell adhesion on implants is essential, on the one hand, in order to properly integrate the foreign material into the tissue in the course of the wound healing process after implantation. On the other hand, cell adhesion can contribute to adverse effects such as thrombus formation[143] or fibrous capsule formation.[144] Cells usually sense their environment via their actin filament based protrusions termed "*lamellipodia*" in fibroblasts and epithelial cells or "*pseudopodia*" in amoebae and neutrophiles.[144] The tails of these protrusions act as feelers that "scan" the ECM or the biomaterial's surface and allow crawling of the cell over the surface in order to proliferate and spread. Actual binding between the surface and the cell is mainly accomplished via integrins, a family of heterodimeric transmembrane proteins located on the cell surface, and distinct, cell adhesive proteins such as vitronectin, fibronectin, or laminin in their proper, active conformation on the substrate surface or within the ECM.[145, 146] Integrins recognize and bind specifically to the tripeptide sequence arginine-glycine-aspartic acid (RGD-motive) displayed by cell adhesive proteins. However, a fine-tuned balance of binding strength has to be achieved in order to permit viability, migration, and proliferation of the cells on the surface because too weak as well as too strong adhesion prohibits cell growth and proliferation as summarized in Table 1.[147] Furthermore,

substrate geometry, surface chemistry (wettability and charge), and topography (roughness and texture) as well as the respective physiological environment influence cell adhesion, viability, and function.[45, 78, 148, 149] These factors are closely related to non-specific protein adsorption including the quantity and type of adsorbed proteins as well as their respective conformation prior to cell attachment. General rules, however, are difficult to deduce from the current cell adhesion studies since various cell lines behave significantly different. It is often observed that cell adhesion and subsequent activity are generally superior on hydrophilic than on hydrophobic substrates.[150, 151] This might be attributed to the activity of the adsorbed proteins and the tightness with which they are adsorbed to the substrate. Hydrophobic substrates promote tight and irreversible adsorption of proteins as well as denaturation which in turn can hinder the required ability of the cells to remodel the extracellular matrix (ECM) during settlement and therefore result in reduced cell adhesiveness.[152]

Table 1. Correlation of the extent of cell adhesion with the subsequent cell behaviour.[153] Arrows indicate permitted (up) and prohibited (down) cell behaviour.

Cell behaviour/ Cell spreading	Viability	Migration	Proliferation	Differentiation
Small 	↓	↓	↓	↓
Medium 	↑	↑	↑	↓
High 	↑	↓	↓	↑

When designing biomaterials basically two approaches have been used to modulate cell substrate interactions. One strategy applies bioinert surfaces such as PEG modified surfaces which prohibit the non-specific protein adsorption and therefore show strongly reduced cell attachment. Therefore, the activation of the body's immune response as well as blood coagulation, extracellular matrix deposition and other interactions between the biomaterial and the surrounding are prevented.[153] Joints and cups of prosthesis, heart valves, and catheters for haemodialysis or scaffolds for therapeutic drug delivery systems are examples for biomaterial applications which are desired to be

highly bioinert. Another strategy focuses on the generation of bioactive biomaterials which specifically respond to the biological environment in a controlled manner by promoting cell attachment, migration, proliferation, and differentiation. Examples for bioactive biomaterial applications are, e.g., a vascular graft prosthesis with a thin layer of non-immunogenic, thromboresistant, endothelial cells,[154] skin substitutes based on polymeric sheets, which are covered by a layer of fibroblasts and keratinocytes,[155] and bone implants, which trigger mineralized, osseous tissue formation at the interface of the implant and the surrounding, native tissue.[45, 156]

In contrast to the controlled either desired or undesired eukaryotic cell adhesion, prokaryotic (bacterial) cell adhesion on implant surfaces is a severe problem in clinical praxis and always undesired since it can result in serious and life-threatening infections. Antibiotic therapy often fails due to antibiotic resistance and the protective slime produced by the bacteria and consequently the surgical removal of the implant is the only efficient therapy. Coagulase Negative Staphylococci (CoNS) are among the most relevant pathogens in catheter related bloodstream infections in hospitals.[157] In addition to CoNS, *Staphylococcus aureus* (coagulase positive), *Pseudomonas aeruginosa*, and *Escherichia Coli* are the most common bacteria diagnosed in infections related to indwelling or implanted foreign bodies.[158] Coagulase Negative Staphylococci strains such as *S. epidermidis*, *S. hominis, S. haemolytius*, and *S. warneri* are usually considered as apathogenic in contrast to Coagulase Positive Staphylococci and are common habitants of human skin. Apathogenic bacteria do not cause infections of healthy humans with intact immune system, however, temporarily immunocompromised patients after surgery are highly susceptible to such infections. Thereby, the bacterial contamination of the implant or medical device can occur via contact with the patient's skin or mucosa during the surgical implantation. Other sources of bacterial contamination are disinfectants or the hospital environment in general. Consequently, biomaterials in contact with the outer part of the body such as urinary tract or intravenous catheters suffer from a higher risk of bacterial infection (0.5-100%) than fully implanted biomaterials (0.1-7%).[159]

Once in contact with the implant or medical device bacteria rapidly adhere and form a monolayer of attached bacteria. Thereafter, many bacterial strains start to secrete a slimy extracellular mixture composed of a polysaccharide hydrogel in which they are embedded and where they can proliferate to generate a biofilm (Fig. 9).[17] This slimy

layer further contributes to tight adhesion of the cell cluster to the implant or the device and protects the bacteria from detachment due to shear stress under flow conditions as well as from antibiotics. Once bacteria have adhered to the surface they recruit further bacteria which can more easily attach via the already secreted extracellular polymeric substances (EPS). Within this assembly bacteria proliferate and recruit further bacteria resulting in a biofilm. Communication of bacteria occurs via quorum sensing by low molecular weight signaling factors termed autoinducers which regulate gene expression of the bacteria in a cell-density dependent manner and hence controls biofilm formation.[160] In addition, some bacterial strains such as *S. aureus* produce and release a multitude of toxins and tissue damaging exoenzymes. After biofilm formation some cells escape from the cell cluster and spread to inoculate new colonization sites, while chronic infection develops when the bacterial cluster reaches a critical size that is large enough to overcome the local host defense. Thereby, the slime around the bacteria makes them often less accessible to the host immune system.

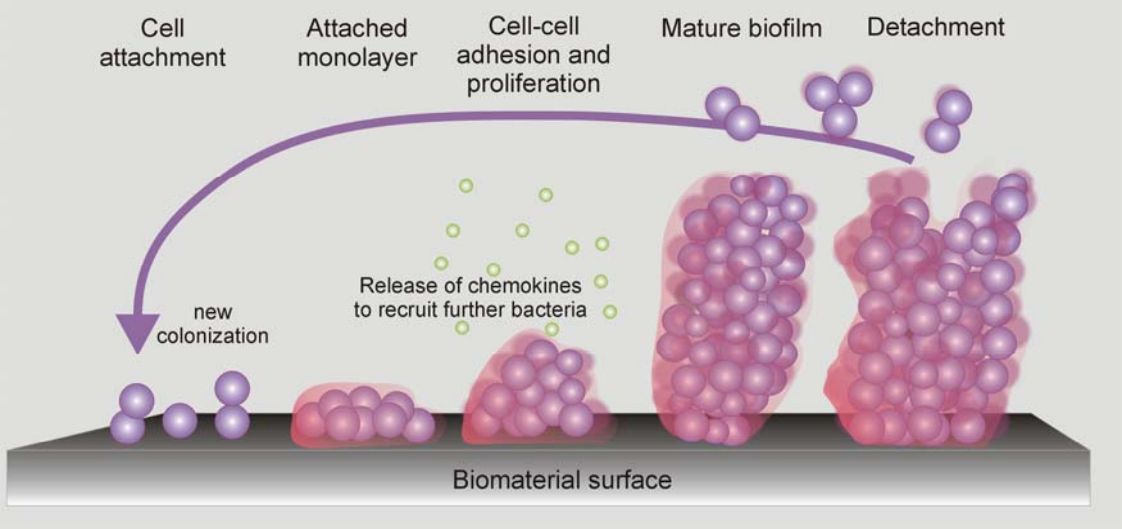


Figure 9. Schematic mechanism of biofilm formation.[17]

In general the adhesion of bacteria to substrate surfaces is a two-phase process comprising an initial reversible physicochemical and a late irreversible molecular and cellular phase.[161] Since bacteria prefer to grow on surfaces rather than in solution they are rapidly attracted to available surfaces via physical forces such as Brownian motion of the surrounding fluid medium, gravitational forces as well as van der Waals, electrostatic and hydrophobic interactions.[159] In addition, signaling factors such as soluble proteins or surface confined aminoacids, peptides and saccharides can recruit

bacteria to the surface via chemotaxis and haptotaxis. In the second time-dependent phase of bacterial adhesion to the surface polymeric structures on the membrane of the bacterium are involved since distinct subunits of these structures act as adhesins. Important polymeric membrane structures of bacteria are capsules which are mostly made up from polysaccharides that protect the bacterium against phagocytosis and desiccation, fimbriae or pili on many Gram-negative bacteria made up from proteins that mediate intercellular or cell-substrate contacts or slime.[159] All of them promote tight adhesion to substrate surface. Factors that determine bacterial adhesion are similar to those of eukaryotic cell adhesion and include substrate chemistry (surface wettability and charge), bacterial properties (surface hydrophobicity and charge), substrate topography (roughness and porosity) as well as the environment of the substrate which enables non-specific protein adsorption. Again generalized guidelines for the design of antibacterial surfaces are hard to conclude due to the huge variety of bacteria with different properties. Surface roughness, e.g., has been demonstrated to have a dramatic impact on bacterial adhesion. While ultrasmooth polymeric surfaces do not allow the adhesion of bacteria, rougher surfaces permit adhesion and biofilm formation.[162]

Strategies to prevent bacterial infection related to biomaterials are to inhibit initial adhesion of microorganisms or rapid killing of the bacteria shortly after adhesion.[19] Therefore, on the one hand, bioinert, antiadhesive surface coatings, in particular, PEG-based coatings are applied.[163-165] On the other hand, bioactive antimicrobial coatings are used such as nanosilver containing[166, 167] or quaternary ammonium groups bearing compounds[168] with known bactericidal properties. For bioactive biomaterials, surfaces have also been modified with covalently or non-covalently attached antibiotics such as vancomycin for a slow release over time.[169] In this respect extensive effort has been made with non-covalently encapsulated antibiotics in a polymeric drug delivery coating, electrostatically or covalently immobilized antibiotics on the biomaterial surface as well as approaches in which the biomaterials substrate itself was used as a drug delivery system for antibiotics. All these attempts suffer more or less from the fact that high amounts of the drug are released initially and therefore sufficiently prevent early on infections but cannot inhibit late infections. Furthermore, antibiotic therapy in biomaterial related infections often fails due to the increasing resistance of pathogenic bacterial strains towards antibiotics.[170] The fact that thrombosis, infection, and inflammation are closely related with each other suggests that biomaterials with short

term protein and cell resistant properties which readily integrate with the host to develop native physiological immune defence in the long term are promising and probably more successful candidates for clinical applications.[18] Hence protein resistant surfaces with enhanced biocompatibility especially in the presence of blood are demanded. Additional resistance of such surfaces towards bacterial adhesion would be advantageous since then the risk of nosocomial infections could be drastically reduced concomitant with a reduced need for antibiotics. This becomes more and more important since nowadays a variety of multiresistant pathogenous bacterial strains have developed by an often excessive and unnecessary application of antibiotics.

1.3 Switchable Surfaces

1.3.1 Non-ionic thermoresponsive polymers

Stimuli-active compounds and in particular polymers have gained increasing interest in the past decades as revealed by the large number of related publications (Fig. 10). Among the group of stimuli-responsive polymers, which respond to external triggers such as pH, ionic strength, solvent, light, and magnetic or electrical fields by a conformational change, are thermoresponsive polymers. The most prominent example of a thermoresponsive polymer is poly(*N*-isopropyl acryl amide) (PNIPAM) (Fig. 11A) which was first mentioned in 1957[171] and rapidly attracted researchers' interest due to its inverse solubility behaviour in aqueous media. In contrast to the common observation that many solutes dissolve easier with increasing temperature, thermoresponsive polymers dissolve in aqueous media at low temperature but become less soluble and eventually precipitate at their respective lower critical solution temperature (LCST). For PNIPAM this reversible phase transition is relatively sharp and located at a temperature of approximately 30 °C to 35 °C with slight variations depending on the molecular weight and the exact microstructure of the PNIPAM polymer.[172-174] Initially this phase transition of PNIPAM was treated as a laboratory curiosity and was studied with purely theoretical, scientific interest. With the emerging awareness of potential applications of the thermoresponsiveness in various fields, however, researchers' attention towards such polymers steadily increased.

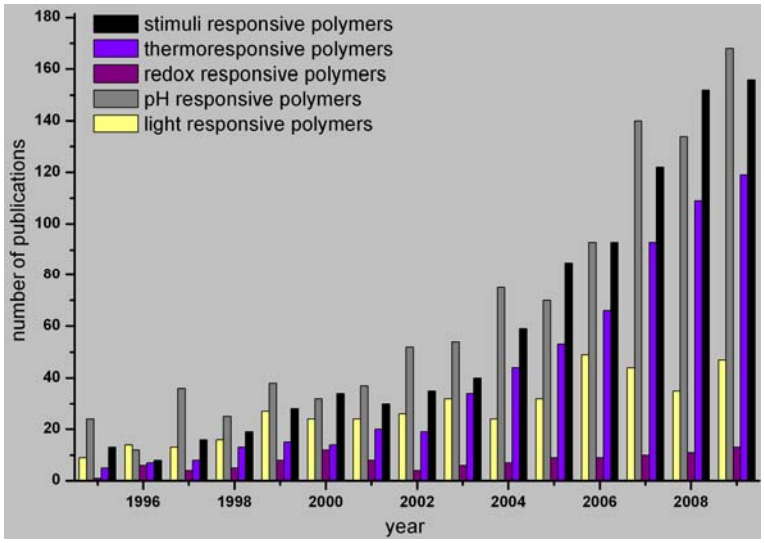


Figure 10. Result of a SciFinder Scholar™ search on stimuli-responsive polymers by different specified search phrases performed in September 2010 (The record for the year 2010 is not displayed due to incomplete coverage).

The phase separation of aqueous thermoresponsive polymer solutions upon heating above their respective LCST can be explained by the hydrophobic effect.[175, 176] In general, any solute has to interrupt or disturb the intrinsic three-dimensional structure of water upon dissolving in aqueous media by means of a rearrangement of the hydrogen-bonded water network. The formation of hydrogen bonds between water and solute molecules leads to an enthalpic gain which contributes to the driving force for dissolution. At the same time, if hydrophobic groups are present in the solute, water molecules have to reorient around these groups. This in turn leads to an unfavorable loss in entropy since the water molecules surrounding the hydrophobic groups lose mobility in terms of translational and rotational freedom. Such a phenomenon is generally termed the hydrophobic effect. Consequently, the free Gibbs Energy ($\Delta G = \Delta H - T\Delta S$) of the solution becomes higher than the free Gibbs Energy of the single components which results in phase separation. Due to such a phase separation thermoresponsive polymers undergo a significant conformational change with a coil-to-globule transition when heated above the LCST. During the phase transition polymer-water contacts are replaced by polymer-polymer and water-water interactions as schematically displayed in Figure 11B for PNIPAM.

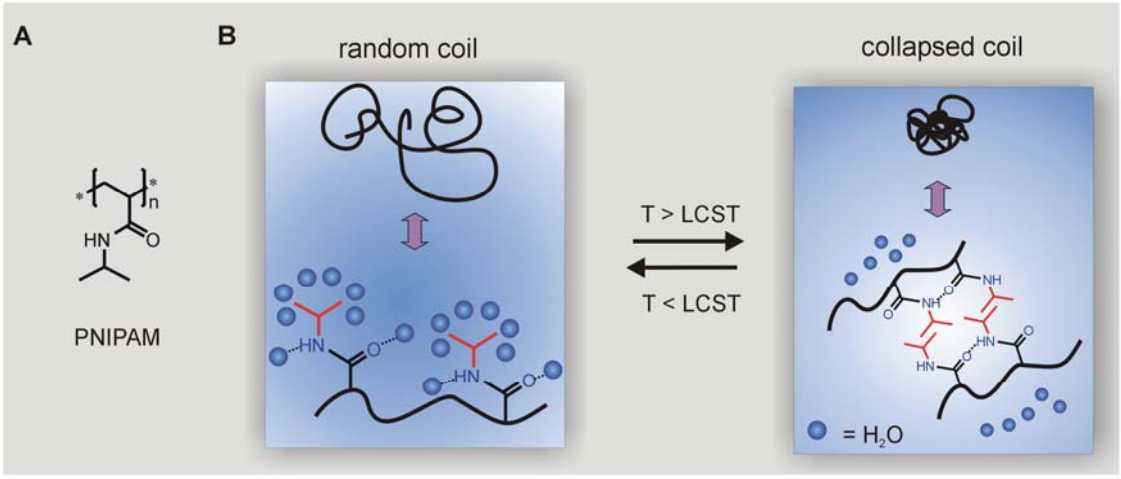


Figure 11. A) Structure of PNIPAM. B) Schematic illustration of the coil-to-globule transition of PNIPAM chains at the LCST in water.

In order to satisfyingly explain the sharp phase transition of LCST polymers the concept of cooperativity of hydration has been introduced.[177] Thereby the hydrogen bonding of water molecules to the polar groups within the polymer is positively reinforced by neighboring repeat units due to the presence of relatively large hydrophobic groups in a thermoresponsive polymer. When a water molecule hydrogen bonds to a polar group,

the polymer chain rearranges and offers space for additional hydrogen bonding with water. Therefore, the polymer in its coiled form below the LCST is believed to be sequentially hydrated along the polymer chain due to these cooperative effects while the sequences are dehydrated at once when heated above the LCST which leads to the observed sharp phase transition.

In general, thermoresponsive polymers can be phenomenologically classified into three types depending on their respective phase diagram.[178] The LCST of type I polymers decreases upon increasing the molecular weight of the polymer. For type II polymers the LCST is hardly affected by the molecular weight and the architecture of the polymer (e.g. linear, branched, star-shaped). In contrast, type III polymers exhibit a bimodal phase diagram with two critical temperatures of phase separation for low and high polymer concentrations, the one dependent the other independent from molecular weight.

Additives such as solvent, salt, or surfactants can shift the LCST of thermoresponsive polymers as they either interfere with the polymer or change the water structure. The effect of salts on the LCST follows the Hofmeister series (Fig. 12) which ranks various ions toward their ability to affect protein hydration and was accessed empirically.[53, 55]

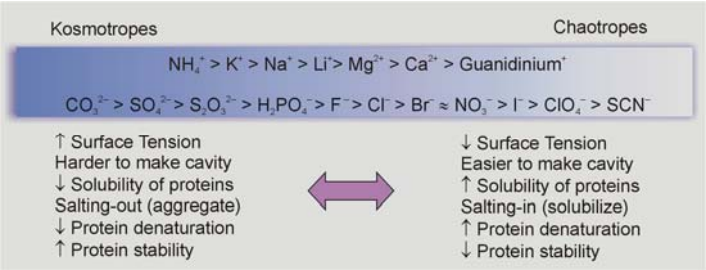


Figure 12. Hofmeister series of ions and their impact on water and proteins (macromolecules).[179]

Thereby, the effect of anions on the LCST is much more pronounced than the one of cations. Kosmotropes are generally defined as ions that are small and have a high charge density and are therefore highly hydrated. Chaotropes are rather big with a low charge density and are therefore much less hydrated compared to kosmotropes. Addition of kosmotropes which interact strongly with water as indicated by a strongly negative free energy of hydration weaken the hydration of the polymer chains by polarizing their inner hydration shell and therefore lower the LCST ("salting-out effect").[180, 181] In contrast chaotropes either increase surface tension of the hydration sphere around

hydrophobic parts of the polymer and therefore lower the LCST or form ionic bonds to the polymer and therefore raise the LCST ("salting-in effect").[180, 181] Sodium dodecyl sulfate (SDS), a commonly used surfactant, increases the LCST, as the hydrophobic, aliphatic tails of SDS interact with the hydrophobic parts of the polymer chain and repulsive electrostatic forces between the negatively charged head groups of the ionic surfactant retard chain collapse and therefore increase the LCST.[182]

Incorporation of a second comonomer, or more generally, the overall hydrophilic/hydrophobic ratio of a water soluble polymer, has a crucial effect on the LCST. While the incorporation of more hydrophobic groups decreases the LCST, the incorporation of hydrophilic groups increases it (see below).

Typically applied techniques to determine the LCST are UV-Vis transmittance measurements at constant wavelength,[183] differential scanning calorimetry (DSC),[184] dynamic light scattering (DLS),[183] and nuclear magnetic resonance (NMR) spectroscopy[183] measurements. Transmittance measurements display the LCST, or more precisely, the cloud point temperature T_{cp} via the optical turbidity of the solution, while DSC indicates the LCST (temperature of demixing T_{dem}) via the endothermic phase transition when hydrogen bonds are disrupted upon polymer precipitation. DLS measures the size of macromolecules or particles via the intensity of scattered laser light, therefore the temperature dependant coil-to-globule transition of single polymer chains can be detected already at very low polymer concentrations by this method. Via proton NMR the coil-to-globule transition of thermoresponsive polymers is indicated by a drastic line broadening of the resonance lines in the spectra at the LCST as the polymer chains become more rigid which shortens the transversal relaxation times (spin-spin relaxation).

Strictly speaking, the LCST can only be determined from phase diagrams at the absolute minimum of the demixing curve. However, within this work the expressions LCST, T_{cp}, and T_{dem} will be used interchangeable to simplify matters.

So far, only PNIPAM was mentioned as the most prominent and most intensively studied thermoresponsive polymer, however, there exist a lot more water soluble, synthetic, thermoresponsive polymers to which all the considerations made above generally apply with small variations depending on the distinct microstructure of the polymer. A brief overview on synthetic, linear, non-ionic homopolymers that exhibit a

LCST in water is given in Figure 13 with representative examples.[178] The largest group of thermoresponsive polymers is made up from amide group bearing polymers. Almost any *N*-alkyl and *N*,*N'*-dialkyl functionalized poly(acrylamide) or poly(methacrylamide) exhibits a LCST in water, however, with a little hysteresis upon heating and cooling, including PNIPAM[172] (**1**), poly(*N*-ethyl acryl amide)[185] (PEAM) (**2**), and poly(*N*,*N*-diethyl acrylamide)[186] (PDEAM) (**3**).

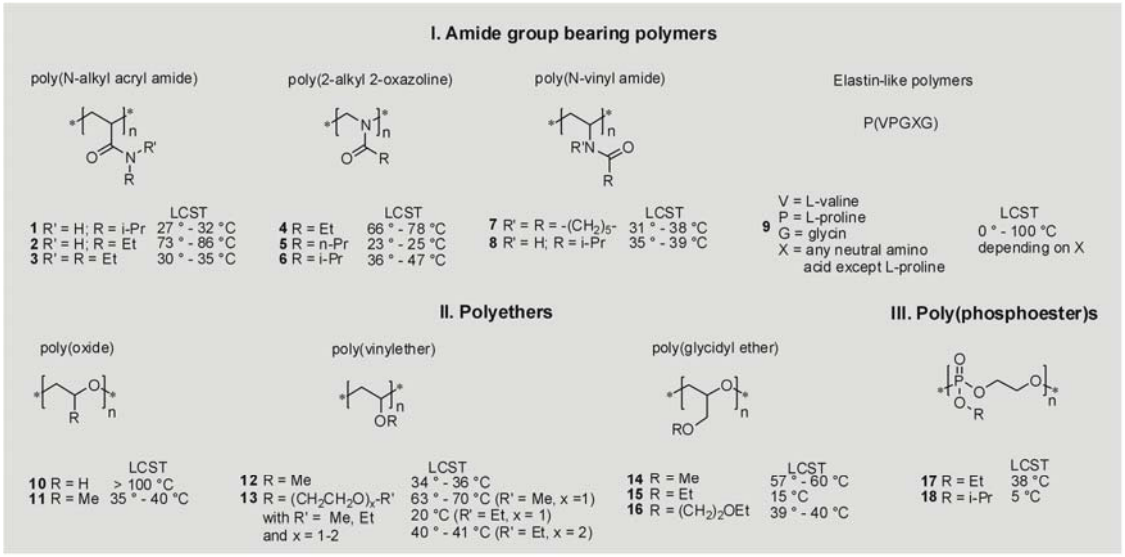


Figure 13. Overview of selected thermoresponsive homopolymers which exhibit a LCST in water with the respective references given in the text besides each polymer.[178]

Another class of thermoresponsive polymers belonging to this group of amide bearing polymers is poly(2-alkyl 2-oxazoline), a structural isomer of poly(*N*-alkyl acryl amide) with the nitrogen located in the polymer backbone. So far, only three derivatives are known to have a reversible LCST in water, this is poly(2-ethyl 2-oxazoline)[187] (PEtOx) (**4**), poly(2-n-propyl 2-oxazoline)[188, 189] (PnPrOx) (**5**), and poly(2-isopropyl 2-oxazoline)[189, 190] (PiPrOx) (**6**). Their phase transitions are very sharp and do not show any hysteresis upon heating and cooling in contrast to PNIPAM. A few representatives of poly(vinyl amide)s which are also amide bearing but with a reversed amide linkage compared to poly(acrylamide)s show thermoresponsive behaviour in water. Among them are poly(*N*-vinyl caprolactam)[191, 192] (PVCL) (**7**) and poly(*N*-vinyl isobutyramide)[193] (PViBA) (**8**), a structural isomer of PNIPAM. In addition to these fully synthetic polymers, there are also bioinspired thermoresponsive polymers based on amino acids such as elastine-like polymers (ELP)s (**9**) which consist of repeating pentapeptides. Elastine, a structure protein in vertebrates, is mainly made up from

alternating hydrophobic and hydrophilic domains while the hydrophobic domains are rich in valine (V), proline (P), and glycine (G) and are responsible for its elastic properties.[194] A common structural motif in the hydrophobic domain is VPGVG, which equals the repeating unit (VPGXG) of synthetic ELP where X can be any neutral amino acid except L-proline.[195] Via the choice of the amino acid X the LCST can be tuned from 0 °C to 100 °C. Thereby, the phase separation of the biopolymer is similar but not equal to fully synthetic amorphous polymers. The biopolymer is in a random coil state below its LCST and turns into a β-spiral when above it accompanied by precipitation.[195-197]

Another group of synthetic, thermoresponsive polymers are polyethers such as poly(oxide)s, poly(vinylether)s, and poly(glycidyl ether)s. Poly(ethylene glycol) (PEG) (**10**) is the only completely water soluble representative of linear poly(oxide)s, whereas poly(propylene oxide)[184] (PPO) (**11**) is only water soluble at room temperature up to a molecular weight of about 3000 g/mol and exhibits a very broad phase transition. A sharper phase transition and no hysteresis upon heating and cooling is observed with different poly(vinylether)s such as poly(2-methoxyethyl vinylether)[198] (PMEVE), poly(2-ethoxyethyl vinylether)[198] (PEEVE), and poly(2-(2-ethoxy)ethoxyethyl vinylether)[199] (PEEEVE) (**13**). In contrast to the latter poly(vinyl methyl ether)[184] (PVME) (**12**) reveals a bimodal phase diagram which is attributed to sequential dehydration of the polymer main chain and the polymer side chains at higher temperature.[200] A relatively new and only rarely studied class of thermoresponsive homopolymers within the group of polyethers are poly(glycidyl ethers). So far only three examples have been identified to show a LCST in water, which are poly(methyl glycidyl ether)[201, 202] (PGME or LPG(OMe) according to the abbreviation used in the following chapters) (**14**), poly(ethyl glycidyl ether)[201, 203] (PEGE) (**15**), and poly(ethoxy ethyl glycidyl ether)[201, 204] (PEEGE) (**16**). These polymers exhibit a sharp phase transition and no hysteresis upon heating and cooling.

The third group of thermoresponsive homopolymers in water is made up from poly(phosphoester)s. Only two derivatives have been reported so far to show a reversible phase transition in water, however, with a small hysteresis upon heating and cooling. These are poly(ethyl ethylene phosphate)[205] (**17**) and poly(isopropyl ethylene phosphate)[205] (**18**).

Since the LCST depends on the molecular weight for type I (e.g. PVCL) and type III (e.g. PVME) polymers as well as on the concentration (for type I-III polymers) of the respective aqueous solution, LCSTs in Figure 12 are mostly given as temperature intervals. Moreover, the given temperature intervals originate from the different techniques (T_{cp}, T_{dem}) used in order to determine the LCST. However, as stated above, actually only one LCST (with the exception of type III polymers) exists, but phase diagrams are not available for all polymers yet.

A common observation is that the LCST decreases with increasing molecular weight and concentration, whereas the polydispersity of the polymer sample affects the sharpness of the phase transition. Figure 14 illustrates examples of UV transmittance curves as they can be observed from thermoresponsive polymer solutions upon heating (dashed line) and cooling (full line). Example (a) displays a relatively sharp phase transition with only a slight hysteresis upon heating and cooling, whereas a broad hysteresis is illustrated in example (b). Such a hysteresis can be attributed to a retarded rehydration of the polymer after temperature induced precipitation due to partial vitrification of the polymeric material in the polymer-rich phase.[206] Such a hysteresis is more pronounced when the glass transition temperature (T_g) of the polymer is higher than the LCST as in the case of PNIPAM. Example (c) illustrates a broad phase transition as it is observed from polymer samples with high polydispersity or no cooperativity effects on hydration due to the absence of hydrophobic groups (like in PEG) which is not very useful for most applications of thermoresponsive polymers.

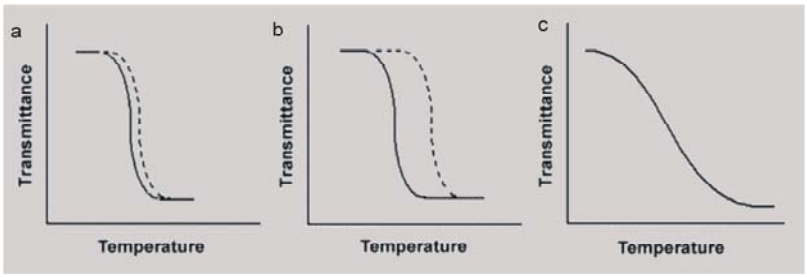


Figure 14. Examples of different UV transmittance curves of thermoresponsive polymers.[207]

Applications of thermoresponsive polymers are diverse including various polymer architectures such as hydrogels via cross-linking or micelles via self-assembly. In addition, thermoresponsive polymers are also applied at interfaces via surface coatings of colloids and soft nanoparticles or flat surfaces for e.g. bioseparation such as purification and concentration of proteins,[208, 209] intelligent drug delivery

systems,[210, 211] sensor technology,[212, 213] textile coatings,[214] diagnostics,[215, 216] and tissue engineering.[217, 218] Since many of these applications are in the biomedical field, biocompatibility of the respective thermoresponsive polymer is a prerequisite for a sustainable and safe application. From the homopolymers shown in Figure 13 PNIPAM[219] (**1**), poly(2-ethyl 2-oxazolines)[98] (**4**), poly(N-vinyl caprolactam)[219] (**7**), ELPs (**9**), PEG[220, 221] (**10**), and poly(phosphoester)s (**17**, **18**) have been reported to be biocompatible polymers with limited confirmation in some case. In general, the presence of an amide group within the polymer is not too favorable for biocompatibility because proteins will interact with these groups via hydrogen bonding.[222, 223] Moreover, PEG as the bioinert benchmark material exhibits a theoretical LCST above 100 °C with a very broad transition which is inappropriate for application. Copolymerization in general is an effective tool in order to adjust the LCST via the hydrophobic/hydrophilic balance and fine-tune it according to the requirements of the respective application. In addition, copolymerization offers a way to introduce further reactive functional groups for additional conjugation or derivatization. To prevent compositional gradients within the copolymer, however, the polymerization coefficients of the comonomers should be equal and around one in order to yield homogenous statistical copolymers. Another benefit of copolymerization is the generation of double responsive polymers which do not only respond on temperature but also to another external trigger either as statistical copolymer or blockcopolymer.[207] For instance, additional pH responsiveness can easily be introduced by incorporation of an ionic comonomer.

1.3.2 Thermoresponsive surfaces

Via tethering thermoresponsive polymers on surfaces the properties of the surface can be altered by a change in temperature. Such surfaces with a solid-water interface are termed smart or switchable and have attracted reasonable attention due to promising applications in the biomedical field, in particular for tissue engineering purposes.[224] Upon the temperature increase the initially extended and hydrated, tethered polymers undergo a phase transition at the LCST similar to the free polymer in solution at which they dehydrate and collapse on the surface as schematically illustrated in Figure 15. The phase transition on the surface is generally accompanied by a change in wettability, thickness, and rigidity of the polymeric coating.

Experimental techniques for studying the phase transition of switchable surfaces are rare and include contact angle measurements,[225] surface force measurements,[226] atomic force microscopy (AFM),[227] quartz crystal micro balance (QCM),[228] neutron reflectometry,[229] and SPR spectroscopy in the quasi static as well as in the real-time mode.[230, 231] In addition to the limited techniques available, surface characteristics of surfaces modified with thermoresponsive polymers such as chain density, molecular weight, thickness, roughness, homogeneity, and distinct microstructure of the tethered polymer chains further complicate the situation and lead to inconsistencies in the observations. For instance, a decrease of the LCST was reported for adsorbed PNIPAM on mica surfaces compared to the free polymer in solution,[232] whereas no LCST at all was observed on PNIPAM grafted silicon surfaces at low grafting density.[233] Controversially, a gradual phase transition over a wide temperature range, in contrast to the sharp phase transition in solution, was reported for PNIPAM modified surfaces at low as well as at high grafting densities and was attributed to cooperative surface effects.[234, 235] So far, no rational guidelines exist in order to precisely control and predict the LCST on surfaces and further comparative and detailed studies are required.

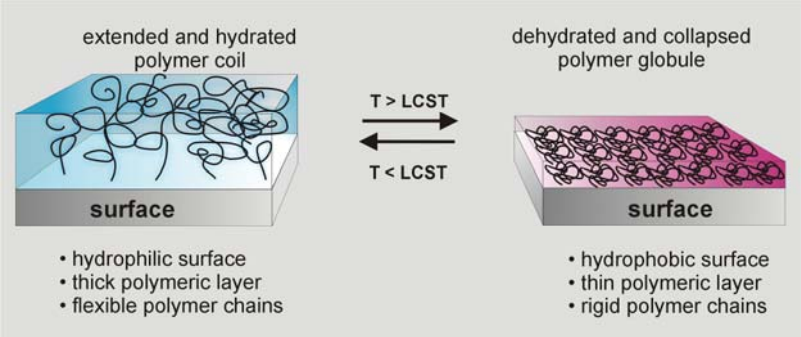


Figure 15. Schematic illustration of a reversible switchable surface.

The phase transition of the thermoresponsive polymer on the surface is accompanied by a wettability change of the surface from more or less hydrophilic to more hydrophobic. This was demonstrated, for instance, with SAMs of poly(ethoxy ethyl glycidyl ether) (**16**) on gold,[225] whereby the extent of the wettability change strongly depends on the chemical composition of the respective polymer and the thickness of the polymer layer. Due to the change in wettability and hydration such surfaces can be used to control protein and cell adhesion. As already outlined in Chapter 1.2 the non-specific protein adsorption is mechanistically explained by the steric repulsion model and retarded by highly hydrated polymer structures. Therefore, proteins adsorb less on surfaces with a

layer of expanded, hydrated polymer chains than on surfaces with tethered, collapsed polymer chains.[230] The degree of adsorption on such surfaces is again dependent on the respective polymer composition and its inherent biocompatibility. Since cell adhesion on surfaces is protein-mediated, cells attach and grow on surfaces which are modified with thermoresponsive polymers above the LCST. By lowering the temperature below the LCST, cells can be easily released from the surface without the use of traditional methods such as mechanical dissociation or enzymatic digestion. The release of cells below the LCST is commonly attributed to the physical force upon rehydration of the collapsed polymer when the chains expand and stretch away from the surface.

It has been shown that cell removal from tissue culture polystyrene (TCPS) by mechanical or enzymatic methods damages both cells and their extracellular matrix (ECM).[236, 237] The ECM is a highly hydrated network which surrounds cells in tissue and contains three major components: fibrous elements such as collagen, elastin, and reticulin, spacefilling molecules such as proteoglycans, and adhesive glycoproteins such as fibronectin, vitronectin and laminin.[238] Communication of cells in tissue occurs via the ECM. ECM proteins interact with integrins, which are receptors located on the cell membrane, and thereby transmit information across the cell membrane into the cytoplasma.[146] Hence, the ECM and the cell surface bear essential proteins which are critical regulators of diverse cellular functions including cell adhesion, growth, and migration. A method which releases cells via a very gentle method by simply lowering the temperature has high potential in tissue engineering, in particular, as whole cell sheets can be released with their ECM remaining intact. Numerous studies with PNIPAM and NIPAM based copolymer coated surfaces have been reported for the temperature triggered release of cells.[218, 239] However, the completion of the release of biomolecules from the surface has only been merely addressed so far. Even small amounts of remaining proteins could limit repeated use of such switchable surfaces as it affects the reversibility on the long term which is essential, for instance, in biosensor technology.[240]

A very interesting and more biocompatible alternative to PNIPAM modified surfaces for controlled cell adhesion are surfaces coated with a copolymer made up of oligo(ethylene glycol) methacrylate (OEGMA) and 2-(2-methoxyethoxy)ethyl methacrylate (MEO_2MA) (Fig. 16).[241] The phase transition of this polymer can be adjusted from 26 °C to 92 °C, thus including the physiological range via the comonomer

ratio.[183] Phase transitions for this copolymer are almost independent from external conditions and very sharp as long as the polymer is prepared via controlled radical polymerization such as atom transfer radical (ATRP) polymerization.[227] ATRP gives good control over the molecular weight and yields polymers with low polydispersity in contrast to common free radical polymerization. Sheardown and coworkers studied the phase transition of this copolymer by neutron reflectometry with a mol fraction of 5% and 15% OEGMA, respectively, on silicon surfaces grafted via surface initiated ATRP.[229] Thereby, both polymers exhibit a LCST on surfaces at around 32 °C and 48 °C, respectively, with the more hydrophilic copolymer expectedly showing a higher LCST.

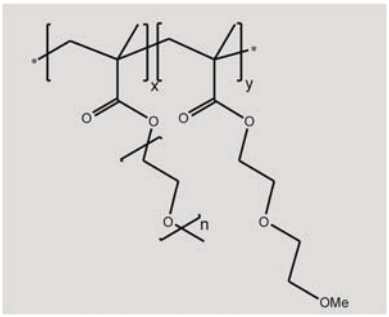


Figure 16. Biocompatible and thermoresponsive OEG-based copolymer for controlled cell adhesion on surfaces.

Both copolymers at the interface contained a water volume fraction of more than 50% below the LCST in their swollen state with a swelling ratio of approximately 1.8 compared to the dry samples. By increasing the temperature close to the LCST the swelling decreased to approximately 1.4, while the polymer layers dehydrated but still contained more than 50% water. By further increasing the temperature to about 18 °C above the respective LCST the polymer chains got even more dehydrated to a final water content of about 20% to 30% and a swelling ratio of 1.2. Thus, the phase transition of such surfaces is also gradual and not sharp. The different states of the tethered polymer chains can be divided into an extended state below the LCST, a compressed state around the LCST, and a collapsed state far above the LCST. The authors further demonstrated that the presence of salts in the surrounding liquid medium has a pronounced effect on the tethered polymers in the extended state according to a "salt-out effect" which leads to a more compressed polymeric layer compared to the extended state of the tethered polymer chains in bare water. In addition, the effect of proteins on the tethered polymer conformation was investigated. Thereby, it was shown

that the polymeric layer on the surface did not interfere with the test protein lysozyme up to concentration of 1 mg/mL protein in buffer both above and below the LCST. The results of protein adsorption studies on these surfaces with lysozyme below and above the LSCT were interesting. At both temperatures remarkable and similar reduction of the non-specific adsorption of lysozyme was observed in comparison to a non-coated bare silcon wafer. This is different from the observations made on PNIPAM[230, 242] grafted surfaces which the authors explain with their observation of the incomplete dehydration of the polymer layer above the LCST. As mentioned above, the polymer chains in their collapsed state still had a water content of more than 20%, which could explain the observed, unusual protein resistance at temperatures above the LCST. However, lysozyme is not a very "sticky" protein and further investigations with fibrinogen as a better and more "sticky" model protein would significantly support the authors' explanation, if similar results would be obtained.

In general, for a deeper understanding of themoresponive surfaces more studies with different thermoresponsive polymers are required which address the phase transition of the polymer in solution in correlation with the observed phase transition of the tethered polymer on surfaces. Thereby flat as well as curved surfaces such as nanoparticle surfaces would be interesting in order to be able to conclude rational design guidelines for swithable surfaces with predictable phase transitions.

1.4 Dendritic polyanions as anti-inflammatory compounds

1.4.1 The inflammatory response

As already outlined in Chapter 1.2, inflammation is a common consequence of the non-specific protein adsorption on artificial materials like implants due to activation and adhesion of platelets which start to form thrombi. The cascade of thrombus formation is closely associated with the inflammatory cascade and vice versa.[16] Furthermore, the presence of endotoxins, which are lipopolysaccharides derived from the outer cell membrane of Gram negative bacteria, on implants or biomedical devices is a serious issue since endotoxins, in contrast to the bacteria from which they originate after cell lysis, are often heat resistant and not affected by common sterilization procedures. Therefore biomaterials often suffer from a contamination with endotoxins despite sterilization. These endotoxins instantaneously lead to antibody development and initiation of the inflammatory cascade when recognized by the human immune system. In general, acute inflammation is the common response of the innate immune system to potentially harmful stimuli such as endotoxins but also pathogens, injury, or foreign bodies. Basically once initiated by a stimulus the inflammation cascade is mainly mediated by dendritic cells, mast cells, and macrophages which reside in the tissue and become activated and therefore release a multitude of different pro-inflammatory signaling factors. Consequently, typical signs of inflammation occur such as enhanced blood flow due to increased vasodilatation which leads to warming and redness of the affected tissue. Moreover, fluid and plasma proteins accumulate at the site of inflammation due to enhanced permeability of the blood vessels which results in swelling of the affected site. In addition, pro-inflammatory signals mediate the expression of cell adhesion molecules (CAMs) on the vascular endothelial surface adjacent to the site of inflammation. Thereby, recruitment of leukocytes (neutrophiles, monocytes, and eosinophiles) from the blood stream to the site of inflammation is promoted according to the chemotactic gradient generated by the residential activated macrophages within the tissue. Once the afflicted site in the tissue is reached leukocytes either eliminate pathogens and foreign bodies or help wound healing of destroyed tissue.

1.4.2 The role of selectins in inflammation

The cascade of leukocyte recruitment from the blood stream to the site of inflammation is mediated via CAMs which are expressed on the vascular endothelium, on leukocytes or platelets. Basically leukocyte recruitment is a receptor mediated process and can be divided into four steps which are schematically illustrated in Figure 17.

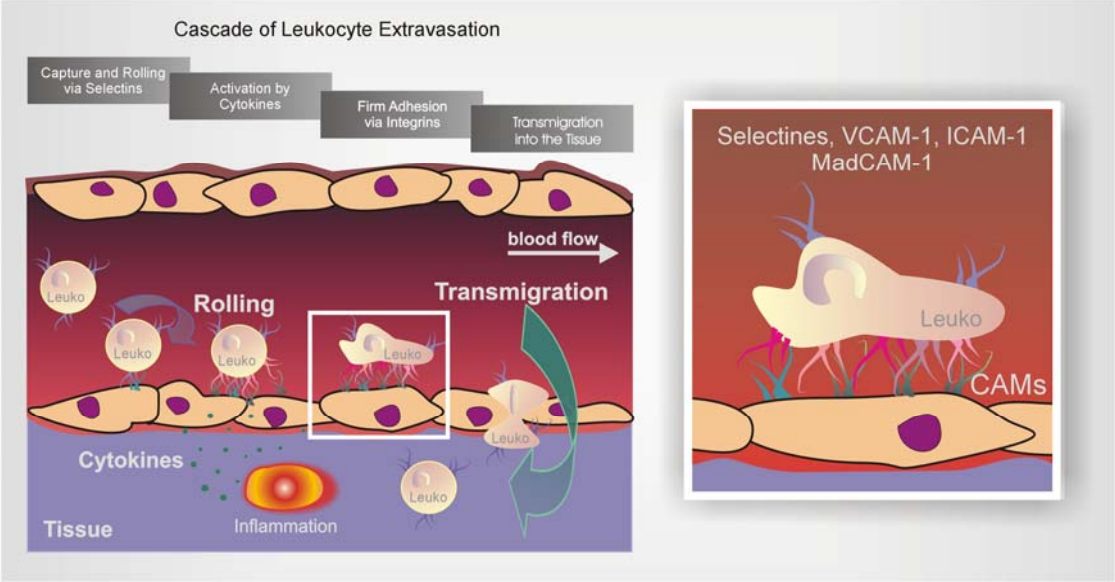


Figure 17. Illustration of leukocyte recruitment and extravasation from the blood stream to the site of inflammation (the amplification in the box on the right hand site highlights certain important cell adhesion molecules (CAMs)).

Upon an inflammatory stimuli pro-inflammatory cytokines, small protein based cell signaling molecules such as tumor necrosis factor-alpha (TNF-α) or interleukin-1 (IL-1) are released which initiate the cascade of leukocyte recruitment. These cytokines bind to their receptors on the endothelium, the extracellular matrix (ECM), and to cellular components of the blood which leads to a transcriptional up-regulation of CAMs (e.g. E-selectin), triggers the release of intracellular stored CAMs (e.g. P-selectin) or transforms CAMs into an active state (such as L-selectin and integrins). Hence leukocytes, which travel with the blood and usually do not bind to the endothelium, can now reversibly and transiently tether to the endothelium via weak selectin-mediated interactions under shear stress at the site of inflammation. Subsequently the leukocytes start to roll on the endothelium and thereby slow down continuously. This selectin mediated capturing and rolling of leukocytes is generally considered to be the first step of the adhesion cascade. In the second step, further chemotractants such as cytokines activate the rolling leukocyte and therefore upregulate the expression of heterodimeric

integrins, which are transmembrane glycoproteins, on the surface of the leukocyte. In addition, their ligands such as vascular cell adhesion molecule-1 (VCAM-1), Inter-Cellular Adhesion Molecule 1 (ICAM-1) or mucosal addressin cell adhesion molecule-1 (MadCAM-1), which belong to the immunoglobulin superfamily, on the endothelium are activated.[243] The subsequent integrin mediated binding results in firm adhesion of the leukocyte to the endothelium in the third step. Finally in the fourth step the adherent leukocyte leaves the blood vessel and extravasates into the inflamed tissue. Secreted extracellular proteases, such as matrix metalloproteinases, pave the way to transmigrate through the junctions between adjacent endothelial cells and the subjacent tissue.[244]

Thereby the family of glycoproteins called selectins is involved in the first step of capturing and rolling of leukocytes on the endothelium.[245] According to their site of expression selectins are subdivided into L-selectin, which is constitutively expressed on leukocytes, P-selectin, which is stored in granules of platelets and in Weibel-Palade bodies of endothelial cells and is translocated to their respective surface upon activation, and E-selectin, which is rapidly expressed on the endothelium upon an inflammatory stimulus.[246] The role of selectins as essential mediators for leukocyte recruitment and extravasation has been demonstrated in studies with knock-out mice which lack selectins or their respective selectin ligands.[247, 248] In such knock-out mice reduced or strongly delayed leukocyte extravasation was observed at sites of inflammation. The structure of selectins is schematically illustrated in Figure 18.

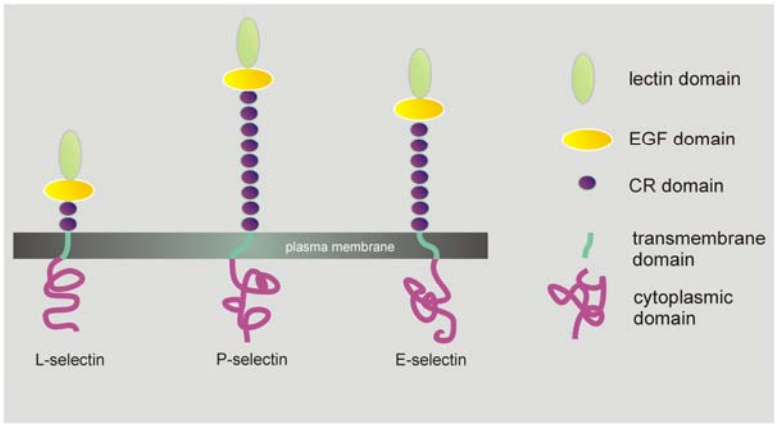


Figure 18. Schematic structure of selectins.

Selectins are made up of five domains and share a high degree of homology. They are composed of a carbohydrate binding, NH_2-terminal, Ca^{2+} dependent (C-type) lectin domain, an epidermal growth factor (EGF) domain, a short consensus repeat domain,

which varies from two to nine repeat units for the different selectins and shows homology with complement regulatory (CR) proteins, a transmembrane domain, and a cytoplasmic domain. The similarity of E- and P-selectin was further demonstrated by means of crystal structures of their lectin and EGF domain in the presence of Ca^{2+} which are highly conserved and reveal a spatial separation of the EGF from the globular lectin domain.[249, 250] Due to the different sites of expression selectins show the highest variation within their transmembrane and intracellular domain.[246] The lectin and the EGF domain are essential for specific binding to the natural selectin ligands.[251] While the actual binding site has been attributed to the lectin domain, it is speculated that the EGF domain supports binding via a proper conformational fixation of the lectin domain.[252]

A variety of specific, physiological selectin ligands is known such as glycosylation dependent CAM-1 (GlyCAM-1)[253] or MadCAM-1[254] for L-selectin, E-selectin ligand-1 (ESL-1)[255] for E-selectin, and P-selectin glycoprotein ligand-1 (PSGL-1)[256] for P-selectin. However, PSGL-1 is less specific and exhibits affinity to all three selectins. So far only PSGL-1 is structurally well characterized while its binding sites with the lectin domain of P-selectin have been identified from crystallographic data upon co-crystallization (vide infra). A simplified illustration of the structure of a PSGL-1 homodimer is given in Figure 19.[246]

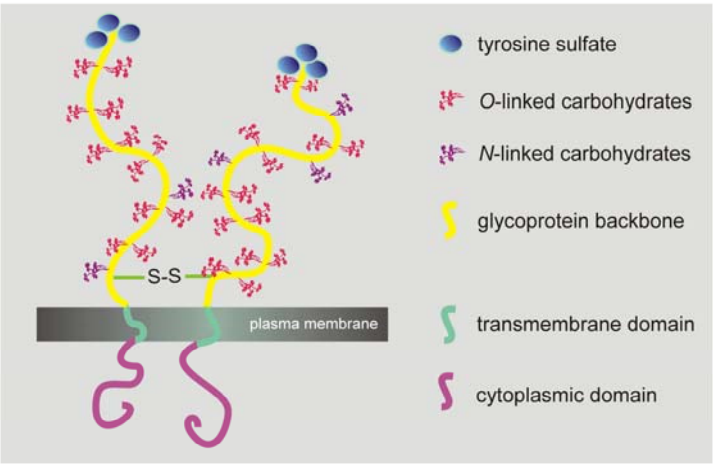


Figure 19. Schematic structure of the physiological P-selectin ligand PSGL-1.

PSGL-1 consists of a short cytoplasmic domain, a transmembrane domain, and a long stretched glycoprotein backbone. The homodimers are stabilized via a disulfide bridge close to the plasma membrane and the carbohydrate moieties are either *O*- or *N*-linked

to the peptide backbone, while the *O*-glycans at the NH_2-terminus are responsible for binding to P-selectin. Furthermore, PSGL-1 bears sulfated tyrosine residues at its NH_2-terminus in close proximity to the carbohydrate binding sites which enhance binding affinity to P-selectin.[249] Despite the lack of detailed information on the structure of the other physiological selectin ligands, it is generally accepted that all ligands for selectins bear a common fucosylated and/or sialylated oligosaccharide binding motif, which is based on the tetrasaccharide sialyl Lewis x ($SiaLe^x$)[257] or structural isomers thereof, such as sialylated Lewis a ($SiaLe^a$)[258] as well as their respective non-sialylated or alternatively in position three of galactose sulfated derivatives (Le^x , Le^a or sulfo-Le^x, sulfo-Le^a) as shown in Figure 20. Therein fucose (Fuc) is indicated in blue, N-acetylated glucoseamin (GlcNAc) in bright green, galactose (Gal) in dark green, and sialic acid (Sia) in red. Based on crystallographic data from co-crystallization experiments of E- and P-selectin with $SiaLe^x$ in the presence of Ca^{2+} distinct binding sites have been identified.[249, 250] A few of them are indicated in Figure 20 for of E-selectin.

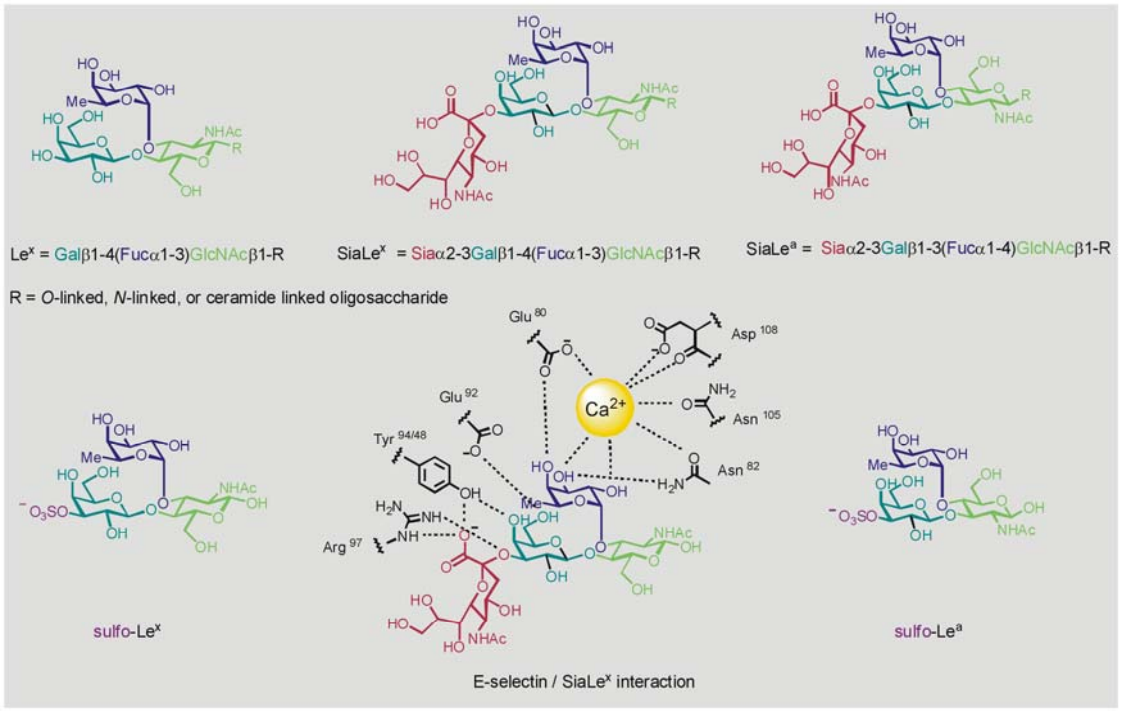


Figure 20. Structure of the common binding motifs of selectin ligands (Le^x, $SiaLe^x$, $SiaLe^a$, sulfo-Le^x, and sulfo-Le^a) and suggested binding sites via Ca^{2+} complexation and hydrogen bonding of $SiaLe^x$ with E-selectin from crystallographic data.

Somers et al. demonstrated that crystal structures of $SiaLe^x$ with E-selectin or P-selectin, respectively, are quite similar and furthermore do not dramatically differ from the crystal structures with the physiological PSGL-1 ligand.[249] However, additional

binding sites for the tyrosine sulfate residues of the N-terminal peptide sequence of PSGL-1 have been identified and it is hypothesized that P-selectin exists in two conformational states, namely, a low affinity state which binds Sia-Le^x and a high affinity state induced during PSGL-1 binding in order to maximize the contact area.[249]

From the solved crystal structure it becomes obvious that mainly the fucose and sialic acid part of SiaLe^x are involved in the binding to P- and E-selectin via Ca^{2+} complexation and hydrogen bonding which likely applies also to L-selectin. However, binding of PSGL-1 to L-selectin and P-selectin bears an additional feature compared to the binding to E-selectin. Both L- and P-selectin binding are sulfate ester dependent while E-selectin binding is not. This is supported by the structure of their respective ligands which reveal sulfate ester groups (sulfated tyrosine residue in case of PSGL-1) in addition to heavily *O*-glycosylated epitopes.[259, 260] While E-selectin binding is not enhanced by the presence of sulfo ester groups as it binds both SiaLe^x and sulfo-Le^x with similar affinity,[261] L- and P-selectin recognize both sulfated and non-sulfated glycans but require a carbohydrate and a sulfate ester motive for high affinity binding.[260, 262, 263] Since it was proven with the solved crystal structure of P-selectin and PSGL-1 that there are two spatially distinct binding sites for carbohydrate and tyrosine sulfate epitopes it is generally suggested that this applies also to L-selectin.

Since selectins are responsible for the initial contact of the leukocyte with the endothelium in the recruitment cascade they have become a promising target for anti-inflammatory therapy in disease related to chronic inflammation.[264-266] In contrast to acute inflammation chronic inflammation often originates from a dysfunction of the immune system. The persistence of an antigen (e.g. in case of rheumatoid arthritis or psoriasis) initiates a continuous pro-inflammatory response and results in excessive uncontrolled infiltration of leukocytes into the tissue which contributes to further tissue damage instead of resolution of the inflammation. In the mid 1990s extensive drug research was performed on the basis of SiaLe^x as the lead structure.[267] However, SiaLe^x itself has only moderate affinity to selectins with K_d values reported in the range of 0.1 mM[268] (for comparison the K_d[249] of physiological PSGL-1 is 778 nM). Various structural derivatization of SiaLe^x did not improve the affinity significantly, thus barely any of these low molecular weight compounds was considered for evaluation in preclinical or clinical trials.[265] A significant improvement of the selectin affinity of SiaLe^x and its derivatives can be achieved by a multimeric presentation of the binding

motif, e.g., on liposomes. On the one hand, this mimics the clustering of glycans as in the physiological ligands and, on the other hand, liposomes can mimic the size of leukocytes, on which some of the natural ligands occur, and thereby offer a large surface area for enhanced contact to the respective binding sites.[269] Simple dimers or trimers of SiaLe^x do not enhance binding efficiently and only yield moderate improvements with three to five times tighter binding per SiaLe^x compared to the monomeric moity.[270, 271] In contrast, a multivalent presentation of SiaLe^x on the surface of liposomes was reported to inhibit E-selectin binding in a competitive cell based assay by five orders of magnitude compared to the monomeric ligand.[269] Bruehl et al. went one step further and prepared postpolymerized bifunctional liposomes with embedded SiaLe^x or sulfo-Le^x analogs (5%), respectively, and distinct anionic head groups in the matrix of the liposomes.[272] Such an assembly mimics the natural ligand PSGL-1 with its *O*-glycan and tyrosine sulfate binding motifs as illustrated in Figure 21. In order to evaluate the effect of the additional anionic head groups on the binding of the liposomes towards L-, P-, and E-selectin they prepared neutral liposomes with hydroxyl head groups in the lipid matrix and cationic liposomes with ammonium head groups in the lipid matrix as controls to sulfate ester and carboxylate head groups.

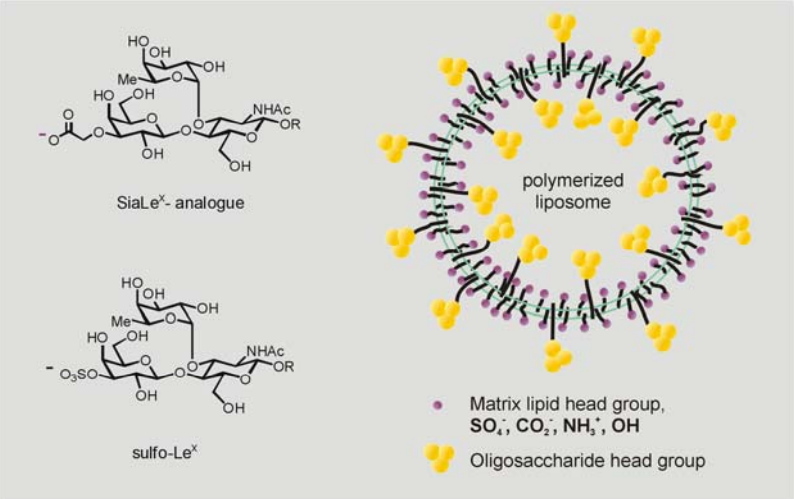


Figure 21. Bifunctional liposomes as multivalent selectin inhibitors mimicking physiological selectin ligands.[272]

The authors could show, by means of a static competitive assay, that compared to the monomeric ligand respectively, the multivalent presentation of the ligand resulted in four orders of magnitude lower IC_{50} values located in the nanomolar range in case of the sulfate ester head groups for all three selectins. IC_{50} values refer to the concentration at

which 50% of the binding of the control is inhibited in a competitive assay. Since IC_{50} values are always in close relation to the set up of the assay, bare numbers are only comparable within one distinct assay. The obtained relative inhibitory potentials are meaningful as long as the setting of the assay is comparable. Nowadays a multitude of different assays for the determination of selectin binding of potential inhibitors is applied which complicates comparison of the efficiency of inhibitors described in literature. In general, selectin assays under shear flow conditions are more appropriate than static assays, since shear stress was found to be a crucial requirement for selectin binding in vivo.[273, 274] Therefore, cell based assays such as flow chamber assays are ideal in vitro set ups in order to evaluate selectin binding or inhibition, respectively, since they mimic the physiological conditions in close approximation. Another appropriate in vitro assay set up under shear stress is displayed in Figure 22.[274]

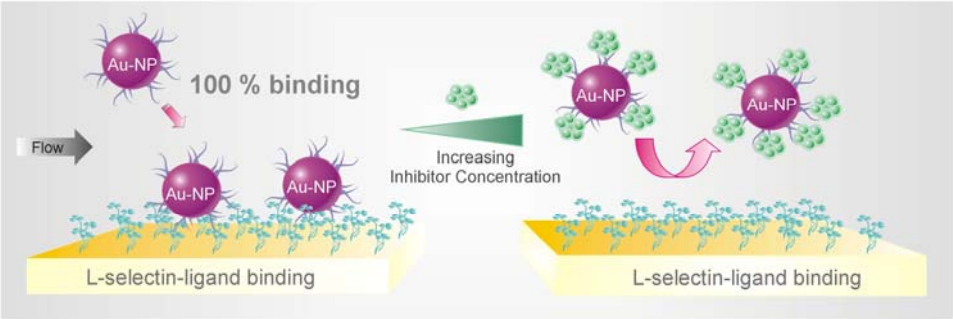


Figure 22. Illustration of a competitive SPR based in vitro selectin binding assay under defined shear stress.

In such a competitive SPR based selectin assay selectin coated Au-nanoparticels (Au-NP) which mimick the leucocyte are passed over a polyacrylamide coated sensor chip surface under defined flow conditions in a microfluidic flow chamber. The sensor chip surface presents the common physiological selectin binding motifs, tyrosin sulfate and $SiaLe^x$, tethered on the immobilized polyacrylamide. In the absence of a potential inhibitor the binding signal from the selectin –ligand (nanoparticle-surface) binding detected via SPR spectroscopy is set to 100% binding and serves as a control value. Subsequently the selectin coated nanoparticles are preincubated with varing concentrations of potential inhibitors and also passed over the sensor chip surface. In case of binding of the potential inhibitor to the nanoparticles a reduced binding signal of the nanoparticles to the sensor chip surface is detected relative to the control value. From a concentration dependent plot of the selectin inhibitor versus % binding IC_{50}

values are accessible, i.e. the concentrations of the respective inhibitor at which 50% binding of the control are observed.

In the latter bifunctional liposome inhibitor study only a static selectin binding assay was applied and the resulting IC_{50} values might not be reproduced by a dynamic assay. However, comparison of relative inhibition values within this study yields stable and valuable results for discussion. While the additional presentation of sulfate esters or carboxylates in case of the $SiaLe^x$ analogue ligand in this study increased binding affinity significantly compared to the neutral (OH) liposomes in the order $SO_4^- > CO_2^-$ for both L- and P-selectin, additional anionic head groups in case of sulfo-Le^x as the multivalent ligand did not increase L- and P-selectin binding compared to the neutral liposome. In case of E-selectin additional presentation of anionic groups within the liposome matrix did not show a pronounced effect for carboxylate head groups and there was only a small improvement in binding in case of the sulfate head groups compared to the neutral liposomes. In contrast, the respective cationic liposomes with ammonium head groups revealed decreased inhibitory potential compared to the neutral liposomes for all three selectins. It is worth mentioning, however, the performance of pure liposomes without sugar moieties, which showed no inhibition for hydroxyl, carboxyl and ammonium head groups but a significant reduction of the IC_{50} value to the low millimolar range for liposomes made up from bare sulfate ester lipids in case of L- and P-selectin. This study highlights the beneficial effect of sulfate ester groups in addition to glycan moieties on selectin inhibition. Thereby the sulfate groups do not necessarily have to be presented on the glycan moiety but may also be presented at spatially distinct positions.[272] The finding that the sulfated lipsome without glycans also has an inhibitory effect on L-and P-selectin clearly demonstrates the electrostatic portion on the selectin-ligand interaction.

1.4.3 Polyanionic selectin inhibitors

Polyanionic sugars such as heparin, heparan sulfate, fucoidan, dextran sulfate, phosphomannon, and sulfatide, a sulfated carbohydrate bearing lipid found in mammalian tissue, (Fig. 23) have long been recognized as selectin inhibitors but were often underestimated for various reasons.[275-278] Heparin is a highly sulfated glucosaminoglycan which is biosynthesized in mast cells and generally isolated from porcine small intestine mucosa as a compound with a complex, not well defined

structure and a broad molecular weight distribution from approximately 3000 to 30,000 g/mol in the case of unfractionated heparin (UFH). Heparan sulfate is structurally related to heparin since they both share a common repeating unit (Fig. 23) but heparan sulfate is exclusively found extracellularly in the ECM or on the cell surface. The animal origin of the latter two bears the risk of disease transmission from animals to humans. Despite this, heparin has been therapeutically used for more than half a century due to its excellent anti-thrombotic and anti-coagulant efficacy. But the strong anti-thrombin-mediated anti-coagulant activity is among the major drawbacks when heparin or analogues are considered as a therapeutic for inflammatory diseases due to the risk of uncontrolled bleeding when applied in high doses on the long term.

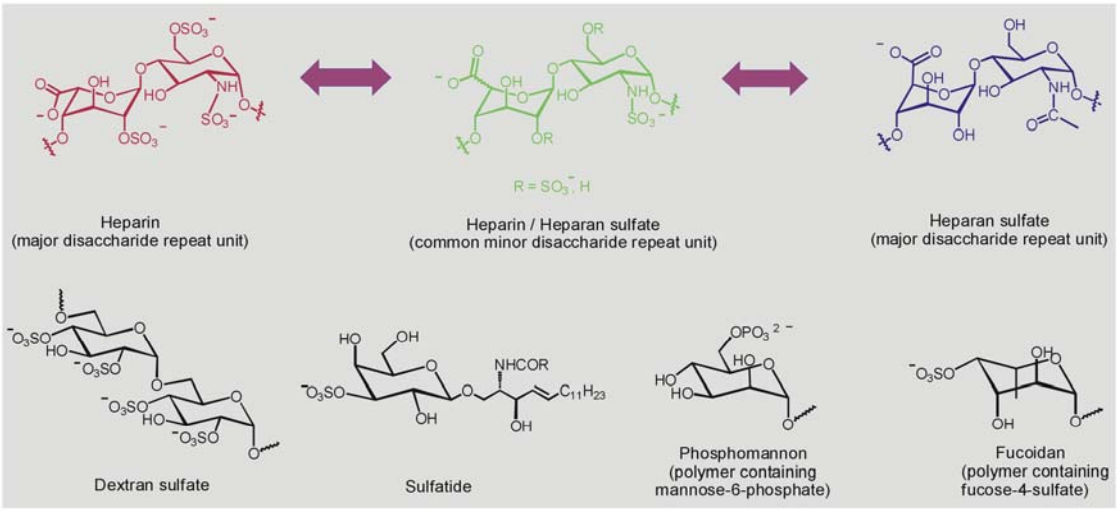


Figure 23. Structure of carbohydrate based anionic L- and P-selectin inhibitors.

Heparin has a variety of biological activities mainly due to its binding to proteins such as antithrombin III, a serine protease inhibitor of thrombin which is responsible for the anti-coagulant effect.[279] Protein binding of polyanions is commonly attributed to ionic and hydrogen bonding interactions which can involve basic or other polar amino acids such as asparagine, glutamine, serine, and others. Typically, the ionic or hydrogen bonding residues are located either directly on the surface or in a shallow binding pocket of the protein where they are presented in a spatially close array. The ability of heparin to block L- and P-selectin was demonstrated to have beneficial effects in cancer therapy where it attenuates cancer cell metastasis.[280, 281] Its polyanionic nature leads to additional anti-viral activity and it was shown that heparin inhibits for instance HIV-1 replication.[282] Furthermore, heparin is commonly used as a surface coating material for

biomedical devices in clinics such as catheters due to its anti-thrombotic activity or as a coating of blood oxygenators in heart-lung machines to enhance biocompatibility.

In order to overcome the mentioned drawbacks of heparin and to gain more specifity of the biological activity non-animal derived, synthetic, or semi-synthetic highly sulfated glycans such as fucoidan (from brown seaweed), dextran sulfate (via sulfation of dextran from bacteria), and phosphomannon (from yeast cells) have been employed and heparin derived analogues with reduced or no anti-coagulant activity have been developed.[283] This latter group of heparinoids includes carboxy-reduced heparin[284] in which the carboxylate group is replaced by a *O*-sulfate group or 2,3-*O* desulfated heparins[285] which retain their L- and P-selectin binding affinity but show reduced anti-coagulant activity after the mentioned chemical modification. Fritzsche et al. have studied different semi-synthetic sulfated, non anti-coagulant polysaccharides based on phycarin, pullulan, and curdlan (Fig. 24) of different molecular weight and with various degree of sulfation and compared their potential as P-selectin inhibitors with commercial UFH and fractions thereof.[286]

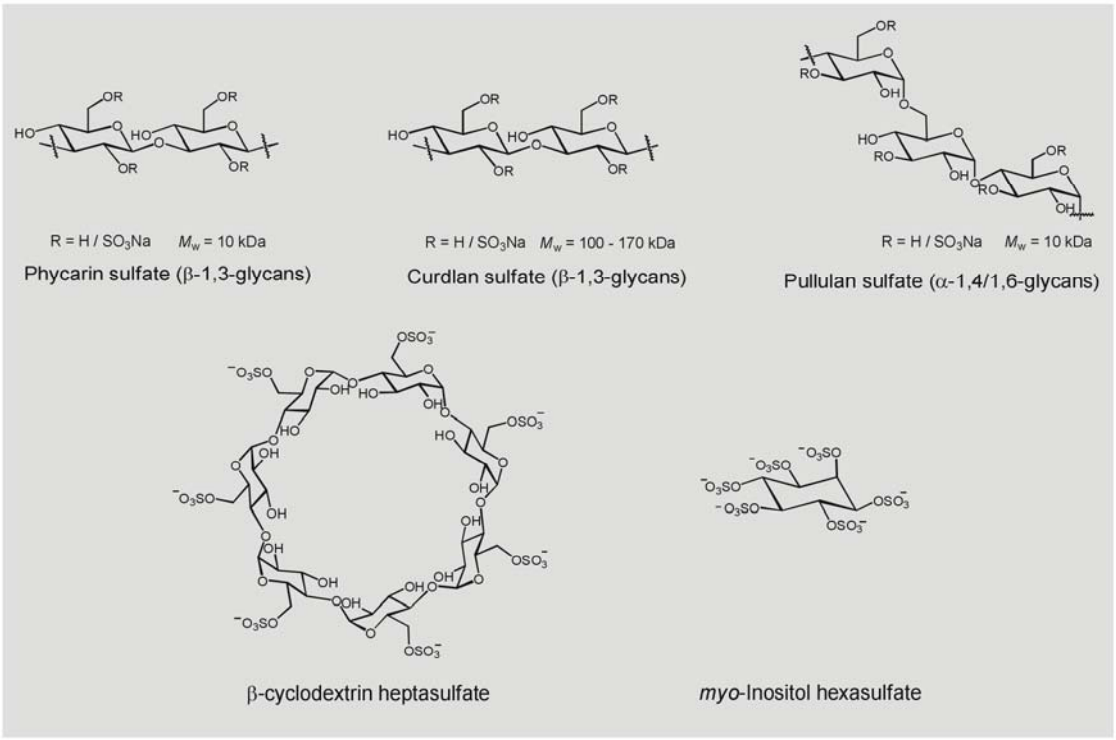


Figure 24. Structures of semi-synthetic carbohydrate based sulfates as well as cyclodextrin sulfate and *myo*-inositol sulfate as selectin inhibitors.

These semi-synthetic glucan sulfates exhibited higher inhibitory efficiency than heparin with a similar degree of sulfation as determined via a competitive cell rolling assay. In

addition, except for the very low molecular weight (M_w = 3.4 kDa) fraction of UFH, all other fractions of heparin showed comparable inhibitory potential to UFH. This suggests that the molecular weight of an inhibitor is of minor importance for its selectin binding potential when above a certain threshold. In line with this finding is the similar performance of phycarin and curdlan sulfates which have a comparable degree of sulfation and an identical carbohydrate structure but differ in size by a factor of ten. Charge density turned out to be much more important for selectin inhibition since with increasing degree of sulfation of the polysaccharide cell rolling on a P-selectin presenting endothelium model surface was significantly reduced while the non-sulfates polysaccharides or the ones with a low degree of sulfation (< 1.5) showed little or no effect on cell rolling velocity. The authors could show that the molecular weight had a modulatory effect on the inhibiton potential since the higher molecular weight polysaccharides required a lower degree of sulfation in order to show the same inhibitory effect as lower molecular weight polysaccharides. This was explained by the enhanced flexibility of the higher molecular weight polysaccharides which can easily present a high local charge density by simple structural rearrangement of the flexible polysaccharide backbone. Such clustering of sulfate groups as a requirement for high affinity binding to L-selectin was also demonstrated on a β-cyclodextrin scaffold and on inositol, a low molecular weight non-classical sugar (Fig. 24).[287] While the non-sulfated compounds did not show any binding affinity, the mono-sulfated cyclodextrin revealed weak and the hepta- sulfated cylodextrin and the hexasulfated *myo*-inositol revealed strong affinity in the millimolar range, however, not as good as fucoidan which was used as a positive control but significantly better than the selectin model ligand $SiaLe^x$. These results further demonstrate the importance of sulfation for high affinity L-selectin binding but more interesting is the performance of the sulfated cyclitol, inositol hexasulfate, which demonstrates that “monosaccharides” – given that cyclitols are considered as non-classical sugars – can also act as selectin inhibitors.

Applying the concept of multivalency[288] Papp et al. prepared a polyvalent selectin inhibitor based on the monosaccharide galactose (Fig. 25).[289] Thereby azide functionalized galactose was coupled to an alkyne functionalized dendritic polyglycerol scaffold via the 1,3-dipolar cyloaddition (click-chemistry). The authors demonstrated that the multivalent display of galactose significantly lowered the IC_{50} values of L-, P-, and E-selectin binding in a competitive in vitro assay[274] (cf. Fig. 22) compared to the

corresponding tetramer of galactose on a pentaerythritol scaffold. In case of L-selectin approximately a 10^3-fold increase in affinity of the multivalently presented galactose over the galactose-tetramer was observed. Furthermore, additional sulfation of the hydroxyl groups of the multivalently presented galactose lowered the IC_{50} values for L- and P-selectin by an additional factor of approximately 100 to the low nanomolar range.

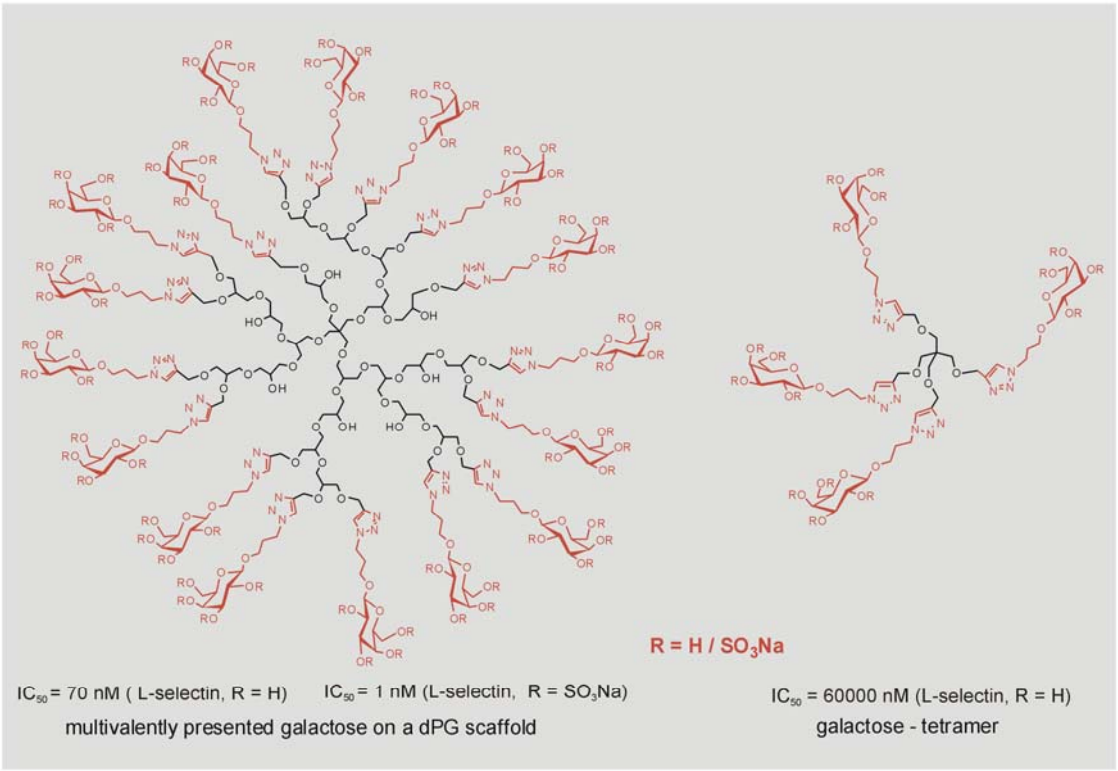


Figure 25. L-, P-, and E-selectin inhibitors based on multivalently presented (sulfated) galactose.

Similar findings were reported by Dernedde et al. who have multivalently presented aminopyrans as sugar mimetics on Au-nanoparticles.[290] Evaluation of their L- and P-selectin binding affinity via a competitive assay revealed no inhibitory effect of monomeric aminopyran while the multivalently presented aminopyran showed an IC_{50} value in the nanomolar range for P-selectin but no inhibiton of L-selectin. However, sulfation of the multivalently presented sugar mimetic yielded efficient inhibitors of L- and P-selectin with IC_{50} values in the picomolar range. In addition to the sugar mimetic the authors also applied Au-nanoparticles that did not have a sugar moiety but simply presented bare sulfate groups on their surface. Such anionic nanoparticles also revealed high affinity towards L- and P-selectin with IC_{50} values in the picomolar range.[290] These results as well as the finding of Bruehl et al.,[272] who prepared sulfate bearing

liposomes as inhibitors for L- and P- selectin and the revealed inhibitory efficiency of sulfated *myo*-inisitol (vide supra), demonstrate that sugar moieties are not necessarily required to achieve L- and P-selectin binding as long as a high density of sulfate groups is presented by the potential inhibitor.

Nevertheless, simple polysulfated and sulfonated synthetic macromolecules have not been systematically tested or applied as selectin inhibitors. However, it was reported that polyvinyl sulfate and poly(anethole sulfonate) as synthetic polyanions exhibit anti-coagulant activities in vitro.[29] In general, such synthetic, non-carbohydrate based heparinoids are considered to be toxic due to long half-life times since they have high molar mass and are not biodegradable.[283] Accidentally, polyacrylates and soluble polyanionic extracts from acidic ion exchange resins such as sulfonated polystyrene based Amberlite™ IR120 or Amberlite™ IRC-84 have been identified as P-selectin inhibitors.[291] When these ion exchange resins were used in the synthesis of $SiaLe^x$ mimetica the determined binding affinities were positively falsified. Another fully synthetic, non-carbohydrate based heparin analogue was reported in 2004 which is much more promising for in vivo applications since it is based on a dendritic polyglycerol[119] scaffold which is a verified, highly biocompatible polymer.[292] The structures of selected sulfated and sulfonated, synthetic polymers which exhibit anti-coagulant activity is given in Figure 26.

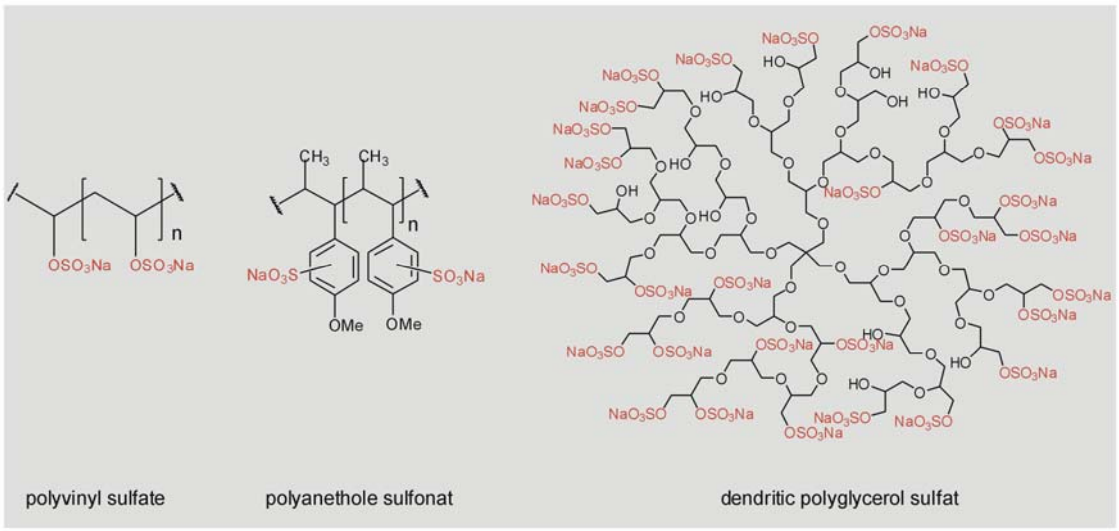


Figure 26. Structure of sulfonated and sulfates synthetic polymers that exhibit anti-coagulant activities.

The anti-coagulant activity of dendritic polyglycerol sulfate (dPGS) was investigated by means of the activated partial thromboplastin time (APTT) assay which is related to the intrinsic coagulation pathway and the thrombin time (TT) assay which refers to the last step of coagulation after addition of excess thrombin when thrombin-mediated fibrin is formed in reference to UHF. While both neutral dendritic polyglycerol and dendritic polyglycerol carboxylates (with a low degree of functionalization, dF = 26%) were inactive in the APTT and the TT assay, dPGS revealed low anti-coagulant activity. The observed higher activity of dPGS in the TT compared to the APTT assay suggests dPGS interferes more in the last step of the coagulation cascade, however, a maximum activity of only 30% of that of heparin was determined via these in vitro assays. Additional complement-induced hemolysis assays including both the classical complement activation (CCA) and the alternative complement activation (ACA) pathway were performed and again showed no anti-complement activity for both the neutral polyglycerol and the carboxylate functionalized polyglycerol. In contrast, dPGS revealed 25 times the anti-complement activity than heparin in the CCA assay, which is a clear indication for the anti-inflammatory potential of dPGS. Therefore further in vitro and in vivo experiments including a structure activity relationship are required to gain a better understanding of the molecular mechanism of the anti-inflammatory effect of dPGS.

2 Objectives

As outlined in the introduction glycerol based polymers exhibit a high potential for applications in the (bio)medical field due to their biocompatible properties.[119] In general, polyglycerols bear multiple hydroxyl groups which can be synthetically converted into various other functional groups and thereby change the properties of the polymer, e.g., hydrophilicity, overall charge, solubility, and many others. Furthermore, biological ligands or surface anchor groups can be immobilized on the polymeric scaffold in order to multivalently present ligands for enhanced binding affinity or in order to covalently tether polyglycerols on surfaces to modify materials' surface properties.

Within the first project linear polyglycerol derivatives (Fig. 27) of different hydrophilicity shall be evaluated for their biocompatible properties on surfaces with respect to protein adsorption, in vitro cell toxicity as well as eukaryotic and prokaryotic cell adhesion in comparison with the common benchmark material polyethylene glycol. Since dendritic polyglycerols have been reported as protein resistant materials[95, 114, 293] in particular the effect of polymer architecture, i.e. branched versus linear, shall be addressed. Gold and glass surfaces offer a good model substrate for evaluation of biocompatibility as outlined in Chapter 1.2.2. Therefore the polymers have to be synthesized with a distinct linker moiety indicated by "X-$(CH_2)_y$-" in Figure 27 for the respective covalent surface immobilization.

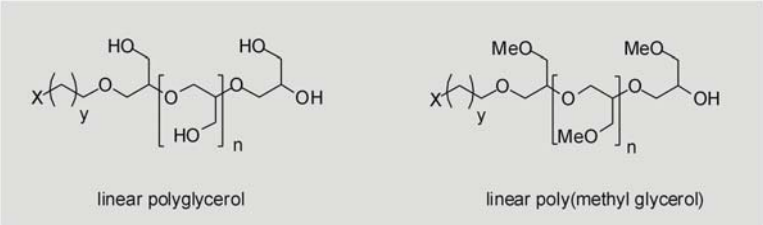


Figure 27. Structure of terminal-functionalized linear polyglycerol and poly(methyl glycerol).

Furthermore within a second project the biocompatible properties of surfaces modified with glycerol based polymers shall be modified such that these properties with respect to non-specific protein adsorption and cell adhesion become switchable upon an external trigger. Therefore, the intrinsic thermoresponsiveness of linear poly(methyl glycerol) (Fig. 27), which exhibits a lower critical solution temperature (LCST) in water at approximately 60 °C could be used.[201] Temperature as an external trigger is

compatible with physiological conditions (e.g. pH, ionic strength) required for protein and cell based applications. However, the LCST of the desired glycerol based polymers has to be adjusted to the physiological range.

Within the third project the potential of dendritic polyglycerol sulfates (dPGS) as L-, P-, and E-selectin inhibitors shall be investigated on the basis of the finding of Türk et al. that dPGS exhibits increased anti-inflammatory potential compared to heparin.[294] Therefore, a preclinical batch for in vitro and in vivo studies has to be synthesized. Moreover, the impact of the degree of sulfation as well as the core size of the applied dendritic polyglycerol on selectin binding affinity shall be addressed. Additionally, the impact of the nature of the anionic group on binding affinity needs to be evaluated. Therefore, dendritic polyglycerol carboxylates (dPGC), sulfonates (dPGSn), phosphonates (dPGPn), bisphosphonates (dPGBP), and phosphates (dPGP) shall be synthesized and their respective selectin binding shall be compared to the binding affinity of dPGS (Fig. 28).

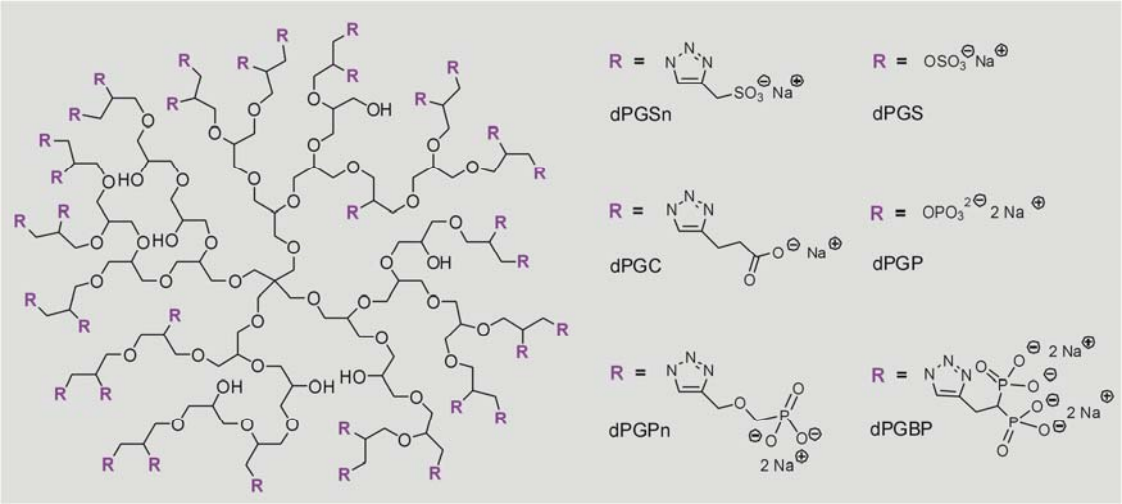


Figure 28. Schematic structure of polyanions based on dendritic polyglycerol.

3 Publications and Manuscripts

3.1 Bioinert surfaces

Within this project the effect of the architecture and hydrophilicity of polyglycerols was evaluated with respect to their biocompatible properties after immobilization on surfaces. Since surfaces modified with dendritic polyglycerol (dPG) and fully methylated dPG have been shown to be resistant towards single protein solutions,[114] the question arose whether their linear analogous will perform the same way or if the observed protein resistance of dendritic polyglycerol and oligoglycerol dendrons[95, 293] is related to their branched architecture. Therefore linear polyglycerols, which bear either hydroxyl groups or methoxy groups in their side chains were prepared with a terminal alkylthiol functionality for self-assembled monolayer (SAM) formation on gold surfaces (cf. Fig. 27). The SAM modified surfaces were characterized and evaluated via IR-reflection-absorption spectroscopy, contact angle measurements, and surface plasmon resonance (SPR) spectroscopy. The latter was used to quantify the relative amount of non-specifically adsorbed proteins from single protein solutions and human serum with a polyethylene glycol (PEG) and a hydrophobic hexadecane thiol reference surface. Furthermore, fibroblast cell adhesion on these modified surfaces was evaluated in reference to a non-modified bare gold surface. The concept of research was provided by Prof. Dr. R. Haag. Research was planned and organized by the author. Synthesis and characterization of the polymers as well as the modified surfaces was perfomed by the aurthor. Initial SPR experiments applying mixed SAMs which were prepared via a reactive anhydride SAM coupling of monoamine functionalized polymers[80] were perfomed by Dr. M. Wyszogrodzka, all further SPR experiments with SAMs of thiol modified polymers were performed by the author. Cell adhesion studies were performed in cooperation with L. Gaetjen, Dr. I. Grunwald, and PD. Dr. A. Hartwig at the Frauenhofer Institute for Manufacturing Technology and Advanced Materials in Bremen (IFAM). The following article (Chapter 3.1.1) was written by the author.

The transfer of the biocompatible properties of glycerol based polymers to more applied surfaces was demonstrated by means of polyglycerol modified glass surfaces. Therefore, dendritic as well as linear polyglycerol derivatives have been modified with a triethoxysilyl tether in order to covalently attach them to activated glass surfaces. The

protein resistance of these modified surfaces was demonstrated by means of fluorescently labeled proteins via epifocal fluorescence microscopy in reference to PEG and hydrophobic hexadecyl triethoxysilane modified glass surfaces. Fibroblast adhesion experiments verified the cell resistant properties of the polyether modified surfaces and additionally demonstrated their long term stability in cell culture. Moreover, bacterial adhesion studies were performed on these modified glass surfaces in cooperation with Dr. K. Schwibbert and Dr. H.-J. Kunte at the Federal Insitute for Materials Reasearch and Testing (BAM) in Berlin. Synthesis and characterization of the polymers was performed by the author and M.Sc. N. Schnurbusch in the course of a practical internship under supervision of the author, glass modification and characterization was performed by the author and M.Sc T. Becherer during a practical internship under the supervision of the author, while evaluation of the biocompatibility (protein adsorption, fibroblast and bacterial adhesion) of these surfaces was performed by the author with support from Dr. K. Schwibbert and Dr. H.-J. Kunte. The concept of research was provided by Prof. Dr. R. Haag and the author. Research was planned and organized by the author. The following manuscript (Chapter 3.1.2) was written by the author.

In addition, the intrinsic thermoresponsivness of linear poly(methyl glycerol) was used in order to prepare biocompatible surfaces which respond on an external trigger by a phase transition. By such a phase transition the properties of the surface can be reversibly altered from a protein and cell resistant to a protein and cell adhesive state. The lower critical solution temperature (LCST) of a thermoresponsive polymer is mainly determined by the hydrophilic to hydrophobic ratio of its chemical composition. Therefore, linear poly(methyl glycerol) was synthetically modified by incorporation of a second more hydrophobic, glycerol based comonomer during the polymerization in order to yield thermoresponsive copolymers with a reversible phase transition in aqueous media in the physiological temperature range (Fig. 29).[203, 295]

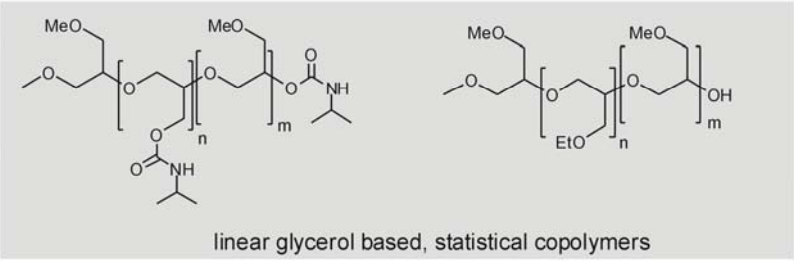


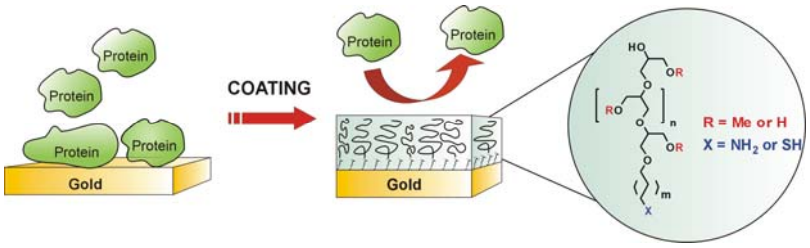
Figure 29. Structure of two thermoresponsive glycerol based copolymers.

Two polymers with different comonomers have been synthesiszed and their respective concentration dependent phase transition under physiological conditions (PBS buffer, pH 7.4, 150 mM ionic stregth) was determined via UV-transmittance measurements. In addition, alkylthiol functionalized copolymers were synthesized for SAM formation on gold and the modified surfaces were characterized via surface IR-reflection absorption spectroscopy and contact angle measurements. The biocompatibility of these surfaces was demonstrated via protein adsorption at ambient temperature applying SPR spectroscopy. Furthermore, the reversible phase transition of the surface confined polymers was evaluated via protein adsorption at different temperatures applying SPR spectroscopy. As a proof of concept the switchability of these surfaces was applied for temperature controlled cell adhesion and detachment from the surface without the use of enzymatic cell detachment agents. The concept and organization of research was developed and performed by the author. Polymer synthesis, characterization, surface immobilization and their characterization, SPR studies and cell experiments were performed by the author and M.Sc. T. Becherer in the course of his master thesis under supervision of the author. The following article (Chapter 3.1.3) was written by the author.

3.1.1 Linear Poly(methyl glycerol) and Linear Polyglycerol as Potent Protein and Cell Resistant Alternatives to Poly(ethylene glycol)

Marie Weinhart, Ingo Grunwald, Monika Wyszogrodzka, Linda Gaetjen, Andreas Hartwig and Rainer Haag*

A coat with arms! A fast and efficient approach to terminal-functionalized linear polyglycerols for subsequent surface modification is described. The resulting biocompatibility of the functionalized surfaces in comparison to a PEG-functionalized surface is demonstrated by protein adsorption and cell-adhesion testing.



This chapter was published in the following journal:

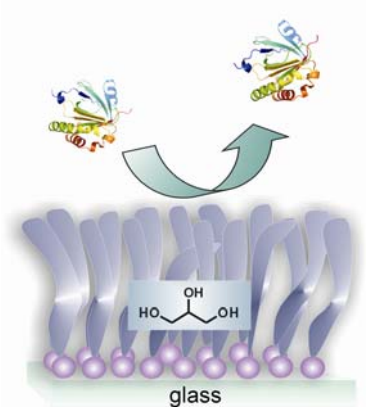
Marie Weinhart, Ingo Grunwald, Monika Wyszogrodzka, Linda Gaetjen, Andreas Hartwig, Rainer Haag, *Chemistry – An Asian Journal* **2010,** *5*, 1992-2000.
(DOI: 10.1002/asia.201000127)

http://onlinelibrary.wiley.com/doi/10.1002/asia.201000127/abstract

3.1.2 Linear and Hyperbranched Polyglycerol Derivatives as Excellent Bioinert Coating Materials

Marie Weinhart, Tobias Becherer, Nicolai Schnurbusch, Karin Schwibbert, Hans-Jörg Kunte, and Rainer Haag *

Glycerol based polymers of linear and branched architecture with a surface reactive silyl tether can be used to generate monolayers on glass. Such modified glass surfaces have excellent protein and eukaryotic as well as prokaryotic cell resistant properties equivalent to PEG.



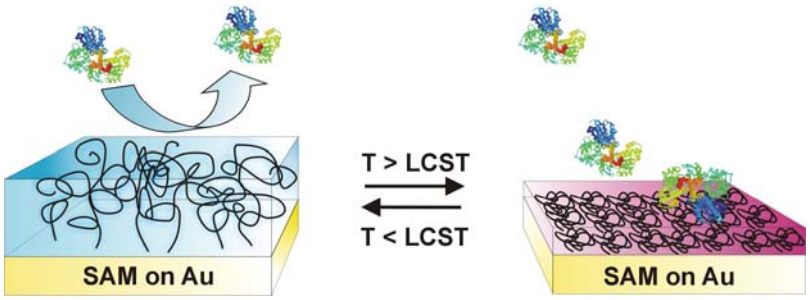
This chapter was published in the following journal:

Marie Weinhart, Tobias Becherer, Nicolai Schnurbusch, Karin Schwibbert, Hans-Jörg Kunte, Rainer Haag, *Advanced Engineering Materials* **2011**, *13*, B501–B510.
(DOI: 10.1002/adem.201180012)
http://onlinelibrary.wiley.com/doi/10.1002/adem.201180012/abstract

3.1.3 Switchable, Biocompatible Surfaces Based on Glycerol Copolymers

Marie Weinhart,* Tobias Becherer, and Rainer Haag

Glycerol based thermoresponsive copolymers to control protein adsorption and cell adhesion on surfaces.



This chapter was published in the following journal:

Marie Weinhart, Tobias Becherer, Rainer Haag; *Switchable, biocompatible surfaces based on glycerol copolymers*, Chemical Communications **2011**, *47*, 1553-1555.

(DOI: 10.1039/C0CC04002A)

http://pubs.rsc.org/en/content/articlelanding/2011/cc/c0cc04002a

3.2 Dendritic Polyanions as Selectin Inhibitors

Within this project the potential of dendritic polyglycerol sulfate (dPGS) as L-, P-, and E-selectin inhibitors was investigated by means of in vitro as well as in vivo studies. Therfore dPGS on varying dPG core sizes and varying degrees of sulfation as well as a low molecular weight triglycerol sulfate reference has been synthesized and characterized. The affinity of dPGS towards L-, P-, and E-selctin was evaluated via a competitive in vitro binding assay applying SPR spectroscopy which yields the respective IC_{50} values. Further experiments were performed applying this assay with L-selectin while the impact of the degree of sulfation and the particle size of dPGS on L-selectin affinity was addressed. The dPGS sample which revealed highest affinity was subjected to a cell based flow chamber assay and verified the concentration dependent inhibitory potential of dPGS on leukocyte-endothelium binding. In addition, this sample was applied in an acute contact dermatitis mouse model with prednisolon® as a commonly administered glucocorticoide as a control. Evaluation was performed by measuring the local tissue swelling at sites of inflammation and the determination of the neutrophil elastase activity, which is an indicator for extravasated leukocytes. Furthermore, the effect of dPGS on the complement system via a mouse model of complement activation was studied in comparison to heparin and dexamethason, a potent anti-inflammatory and immuno suppressive glucocorticoide, as controls, respectively, and the levels of proinflammatory anaphylatoxine C5a have been determined time-dependently. The results suggest that dPGS is a potent anti-inflammatory agent which works via at least two distinct modes of action. The concept of research was mainly provided by Prof. Dr. R. Haag and Dr. J. Dernedde. Synthesis and characterization was perfomed by the author, in vitro studies have been performed in cooperation with Dr. S. Enders, Dr. J. Dernedde, and Prof. Dr. R. Tauber at the Charité – Benjamin Franklin Campus in Berlin, in vivo studies were performed in cooperation with Dr. A. Rausch, Dr. A. von Bonin, and Dr. U. Zügel at Bayer-Schering Pharma in Berlin as well as Dr. K. Licha and Dr. M. Schirner at mivenion GmbH in Berlin. The article (Chapter 3.2.1) was written by Dr. J. Dernedde.

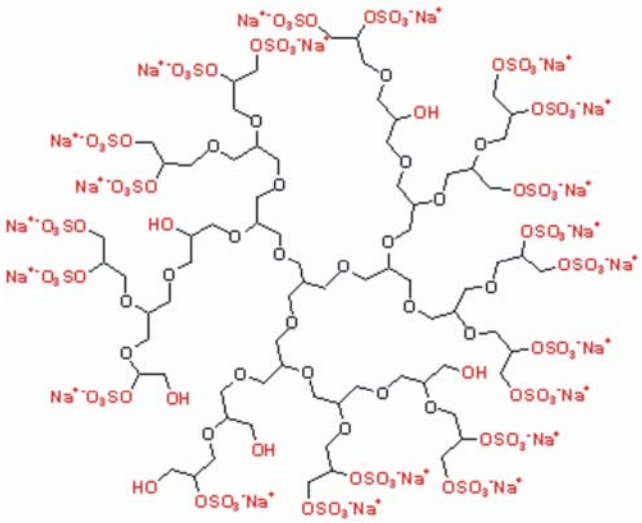
In addition, the impact of the degree of sulfation, the dPGS particle size, and the effective charge of dPGS on L-selectin binding was further investigated by means of various dPGS particles ranging from about three to twenty nanometers. L-selectin binding was evaluated with a SPR based competitive binding assay. The determined

IC_{50} values were correlated with the measured particle sizes and zeta potentials under buffered solutions resembling the conditions of the biological experiments. Three dPGS samples have been applied to a cell based flow chamber assay which is closer to the physiological situation in order to support the IC_{50} values determined via the SPR assay. The concept of research was provided by Prof. Dr R. Haag, Dr. J. Dernedde and the author. Synthesis and characterization was mainly performed by the author, DLS and zeta potential measurements were mainly performed by M.Sc. D. Gröger, whereas the SPR and flow chamber assay was performed in cooperation with Dipl. Biochem. S. Riese, Dr. S. Enders, and Dr. J. Dernedde at the Charité – Benjamin Franklin Campus in Berlin. The manuscript (Chapter 3.2.2) was written by the author.

Furthermore, the impact of the nature of the anionic group on binding affinity was evaluated. Therefore, a versatile approach for the synthesis of highly functionalized dendritic polyglycerol anions and their respective low molecular weight ionic precursors was established. Dendritic polyglycerol carboxylates, sulfonates, phosphonates, and bisphosphonates were synthesized via "click chemistry" of dendritic polyglycerol azide and the respective ionic alkyne. Dendritic polyglycerol phosphates were prepared via direct hydroxyl group interconversion in analogy to the synthesis of dPGS. The affinity of these polyanions towards L-selectin binding was evaluated by means of a SPR based competitive binding assay. The determined IC_{50} values were correlated with the measured particle size and effective particle charge under conditions resembling those of the biological evaluation. The concept of research was provided by Prof. Dr. R. Haag, Dr. J. Dernedde and the author. Synthesis and characterization was performed by the author and M.Sc. D. Gröger in the course of a practical internship under supervision of the author. DLS and zeta-potential measurements were mainly performed by M.Sc. D. Gröger in the course of his PhD work, whereas selectin affinity of the polyanions was evaluated in cooperation with Dr. S. Enders and Dr. J. Dernedde at the Charité – Benjamin Franklin Campus in Berlin. The manuscript (Chapter 3.2.3) was written by the author.

3.2.1 Dendritic Polyglycerol Sulfates as Multivalent Inhibitors of Inflammation

Jens Dernedde, Alexandra Rausch, Marie Weinhart, Sven Enders, Rudolf Tauber, Kai Licha, Michael Schirner, Ulrich Zügel, Arne von Bonin, and Rainer Haag*



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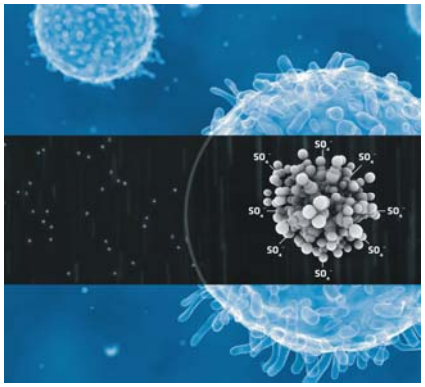
Jens Dernedde, Alexandra Rausch, Marie Weinhart, Sven Enders, Rudolf Tauber, Kai Licha, Michael Schirner, Ulrich Zuegel, Arne von Bonin, Rainer Haag; *Proceedings of the National Academy of Sciences* of the United States of America **2010**, *107*, 19679-19684.
(DOI:10.1073/pnas.1003103107)

http://www.pnas.org/content/107/46/19679.abstract

3.2.2 The Role of Dimension in Multivalent Binding Events: Structure-Activity Relationship of Dendritic Polyglycerol Sulfate Binding to L-selectin in Correlation with Size and Surface Charge Density

Marie Weinhart, Dominic Gröger, Sven Enders, Sebastian B. Riese, Jens Dernedde, Rajesh K. Kainthan, Donald E. Brooks, Rainer Haag*

Polysulfated, flexible inhibitors to L-selectin are investigated. In inflammation selectins mediate leukocyte recruitment from the blood stream to the vessel wall. Therefore, they have evolved into a promising target in anti-inflammatory therapy. Size and surface charge density of the polyelectrolytes is correlated with their efficiency to multivalently bind to L-selectin and hence act as inhibitor in a competitive in vitro assay.



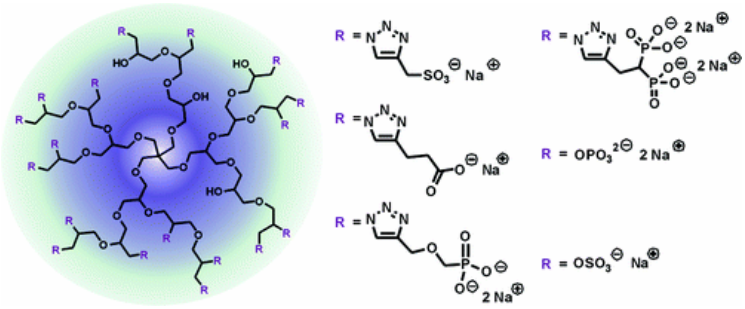
This chapter was published in the following journal:

Marie Weinhart, Dominic Gröger, Sven Enders, Sebastian B. Riese, Jens Dernedde, Rajesh K. Kainthan, Donald E. Brooks, Rainer Haag, *Macromolecular Bioscience* **2011**, *11*, 1088–1098.
(DOI: 10.1002/mabi.201100051)

http://onlinelibrary.wiley.com/doi/10.1002/mabi.201100051/abstract

3.2.3 Synthesis of Dendritic Polyglycerol Anions and Their Efficiency Toward L-Selectin Inhibition

Marie Weinhart,[§‡] Dominic Gröger,[§‡] Sven Enders,[†] Jens Dernedde,[†] Rainer Haag[§]*



This chapter was published in the following journal:

Marie Weinhart, Dominic Gröger, Sven Enders, Jens Dernedde, Rainer Haag,
Biomacromolecules **2011**, *12*, 2502-2511.
(DOI**:** 10.1021/bm200250f)

http://pubs.acs.org/doi/abs/10.1021/bm200250f

4 Summary and Conclusions

In the first part of this work glycerol based polymers of linear and dendritic structure were synthesized and evaluated with respect to their application as coating material for bioinert surfaces on gold and glass substrates (Fig. 30). It was demonstrated that linear poly(methyl glycerol) (**1**, LPG(OMe)) as well as linear polyglycerol (**2**, LPG(OH)) SAMs on gold surfaces exhibit similar protein resistance with single protein solutions as previously reported SAMs of dendritic polyglycerol[114] (**3**, dPG) or PEG[108] with similar molecular weight.

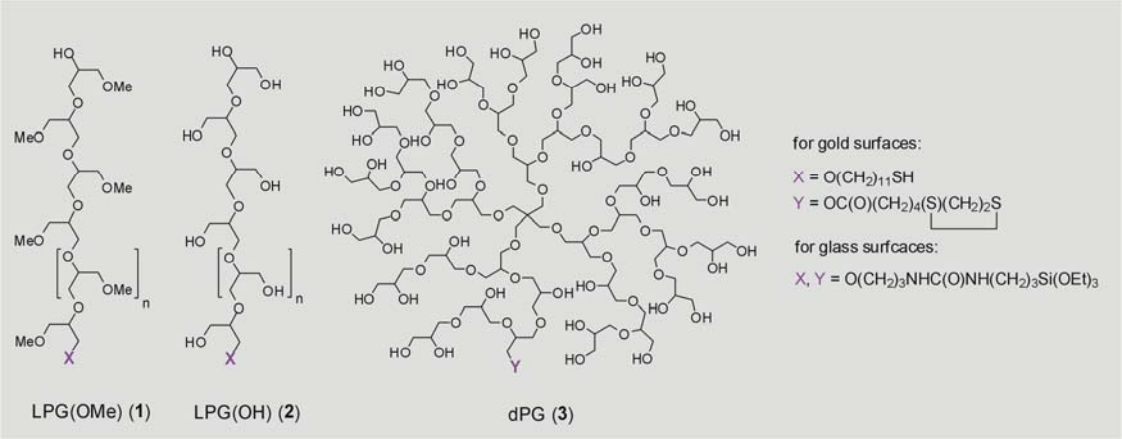


Figure 30. Structures of glycerol based polymers for bioinert surface modification.

However, not any of these surfaces modified with linear polyethers was completely resistant towards protein adsorption from non-diluted human serum. Protein adsorption from such complex mixtures increased in the order LPG(OH) (17% of a protein monolayer) > LPG(OMe) (21% of a protein monolayer) > mPEG (33% of a protein monolayer). It might be speculated that the stronger reduction of LPG(OH) and LPG(OMe) is due to a higher content of internally bound water within the SAM since LPG(OH) and LPG(OMe) probably form less ordered monolayers with lower lateral chain density than mPEG SAMs due to the presence of additional space demanding side chains. For surfaces modified with OEGMA based bottle brushes excellent non-fouling properties also in the presence of complex protein mixtures was reported in literature.[48] This was explained by higher chain flexibility and better surface shielding by the immobilized bottle brushes as opposed to single linear polymer chains as well as a higher amount of internal water. Hence, the possibility for a higher amount of internally

bound water within the SAM of LPG(OH) and LPG(OMe) might lead to the observed reduced protein adsorption from human serum compared to mPEG SAMs.

Despite this, eukaryotic cell adhesion as studied with fibroblast cells was drastically reduced for all surfaces modified with LPG(OH), LPG(OMe), mPEG, and dPG irrespective of the applied substrate on both gold as well as glass when the chain density of the SAM was sufficiently high. In addition, the long term stability of these surfaces was evaluated in the course of the cell studies and revealed all surfaces to remain intact even after one week in cell culture. In vitro cell toxicity data of linear polyglycerol and linear poly(methyl glycerol) further add to the argument that these linear glycerol based polyethers are a good alternative to commonly used PEG.

By changing the substrate surface from gold to glass it was demonstrated that these glycerol based polymers can also be applied on more challenging substrates since comparable protein and cell resistant properties were obtained although the lateral chain density of SAMs on glass is lower than on gold. These results are promising for the modification of polymeric substrates with glycerol based polymers on which high grafting densities are hard to realize. Furthermore, protein adsorption on polyether modified glass surfaces merely increased with the immersion time up to 24 hours. Preliminary static bacterial adhesion studies on these surfaces support glycerol based polymers as bioinert surface coating materials since adhesion of gram-negative as well as gram-positive bacteria was reduced by approximately 99% compared to non-functionalized bare glass. In summary, glycerol based polymers offer an equivalent alternative to PEG as a bioinert coating material. Thereby the hydroxyl bearing polyglycerols allow for further ligand attachment to initiate defined specific interactions with biomolecules or cells. This is especially important for bioanalytical applications such as biosensors where a specific interation is required whereas at the same time a reduced background signal from non-specific interactions is warranted for an improved signal to noise ratio.

Moreover, linear thermoresponsive glycerol based copolymers were prepared with adjustable lower critical solution temperature (LCST) in aqueous media depending on the comonomer ratio. Monothiol functionalized copolymers were immobilized on gold substrates via SAM formation. These copolymers showed good protein resistance at ambient temperature but significantly increased protein adsorption at elevated temperature in the physiological range due to a temperature triggered phase transition.

This phase transition was demonstrated to be reversible for the free polymers in solution as well as for the tethered polymers on the surface and was applied for a temperature triggered release of adherent cells from the surface. Therefore cells were grown on the coated surfaces at 37 °C under common cell culture conditions when the polymeric chains on the surface were collapsed and in their protein adhesive state and released from the surface by simply lowering the temperature. Due to the phase transition of the polymer chains into their well hydrated non-adhesive state the cells detached from the surface with their ECM left intact since no enzymatic treatment was required. Therefore, such bioinert switchable surfaces offer high potential for application as temperature responsive matrix for tissue engineering.

In the second part of this work highly functionalized polyanions based on dendritic polyglycerol were synthesized and evaluated with respect to their efficiency as L-selectin inhibitors (Fig. 31). Initially dendritic polyglycerol sulfate (dPGS) was described as fully synthetic heparin analogue which exhibits reduced anti-coagulant but higher anti-inflammatory properties compared to heparin.[292]

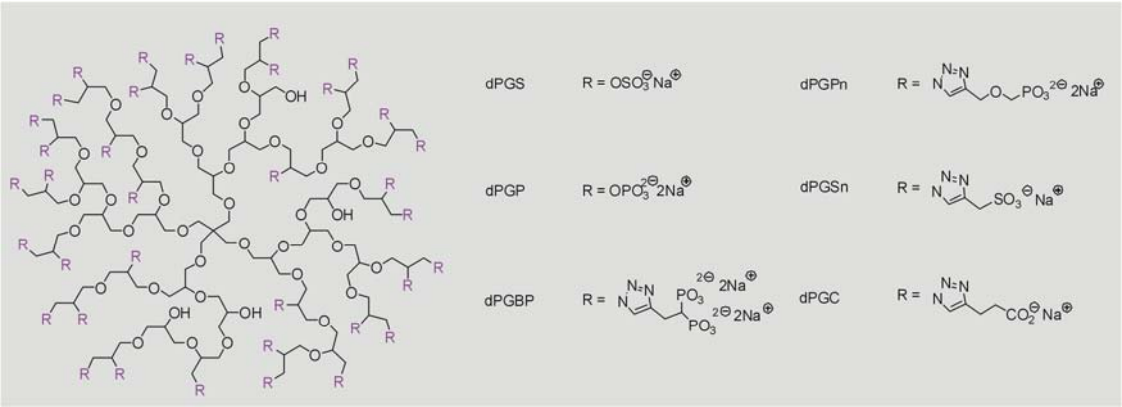


Figure 31. Structure of dendritic polyglycerol anions.

Based on these results dPGS was tested for its ability to bind to selectins which are essential mediators in the cascade of leukocyte recruitment in inflammation. High affinity for L- and P-selectin was demonstrated whereas no affinity towards E-selectin was found via a competitive binding assay in vitro. Thereby IC_{50} values in the low nanomolar range where obtained which were one order of magnitude lower than the ones determined for heparin. The dose dependent anti-inflammatory potential of dPGS was further demonstrated in vivo in an acute dermatitis mouse model which revealed dPGS to be as efficient as the commonly applied glucocorticoid prednisolon®.

Moreover, a structure activity relationship from various dPGS samples of different size and nominal charge density and their binding affinity towards L-selectin was established. Both size and charge density increase the binding affinity towards L-selectin while a bigger size of the sulfate presenting scaffold can compensate to some extent for a lower amount of nominal charges. Size and charge density dependent L-selectin affinitiy of dPGS was further approved via a cell based flow chamber assay which is closer to the physiolocical situation. In order to rule out the effect of the chemical nature of the respective anion dendritic polyglycerol phosphate (dPGP), bisphosphonate (dPGBP), phosphonate (dPGPn), sulfonate (dPGSn), and carboxylate (dPGC) in addition to dPGS have been synthesized (Fig. 31). The study of L-selectin affinity of dendritic polyanions with different chemical nature of the anion revealed an increased affinity in the order carboxylate < phosphate < phosphonate ≈ sulfonate < bisphosphonate < sulfate. Since the binding of polyanions to selectins is mainly electrostatic in nature the observed differences in binding efficiency are not entirely clear but the tendency towards higher affinity with increasing acidity of the anionic group might be derived. Additional DLS and zeta potential measurements under conditions resembling the ones of the biological experiments of these polyanions do not simply correlate with the L-selectin binding affinity. However, a significant difference between dPGS and all the other tested polyanions was observed. dPGS revealed the smallest particle size as well as the lowest zeta potential although all polyanions were synthesized from the same dPG core with well comparable high degrees of functionalization. This suggests that conformational changes within the polymer might occur. This could include backfolding of anionic groups on the surface as well as compression of the dPG backbone to various extents depending on the chemical nature of the anionic group and the surrounding medium. In conclusion, dPGS is a promising polymeric therapeutic for diseases related to inflammation and therefore a strong candidate for a clinical trail.

5 Outlook

From a mechanistical point of view it would be interesting to evaluate the amount of internally bound water and ions within a SAM of LPG(OMe) and LPG(OH) in comparison to a mPEG SAM on gold. For mPEG SAMs the water content of the interface has already been determined by Grunze et al. via neutron reflectometry.[296] This revealed a densely packed alkanethiol based PEG SAM on gold to be less penetrated by water whereas a more loosely packed amorphous monolayer had significantly more internally bound water within the SAM. Since SAMs of PEG with moderate packing density are more resistant towards protein adsorption than densely packed SAMs the internally bound water is thought to be responsible for the observed protein resistance. A comparison of the amount of bound water within the SAM of glycerol based polymers and mPEG could help to explain the observed differences in adsorption from complex protein mixtures.

Furthermore, modification of polymeric biomaterials with glycerol based polymers and proof of their bioinertness and in particular haemocompatibility would be desirable. Therefore polyurethanes as the common catheter material or polyvinylchloride as the common material for blood storage bags are most relevant. In addition, non-adhesive, bacteria resistent surfaces are highly demanded in clinics. This is especially important for surfaces of medical devices which are used repeatedly with various patients such as cardiopulmonary bypass in order to reduce bacterial disease transmission. However, a simple non-adhesive surface is often not sufficient for long term applications and additional bactericidal components are commonly added to the coating material such as antibiotics, nanosilver or quaternary alkylammonium groups.[89] Such “hybrid” surfaces would be desirable since they do not just passivate the surface but also actively kill adherent bacteria.[297]

For a more detailed understanding of the L-selectin binding of the different dendritic polyanions it would be helpful to determine their respective pK_a values under the conditions of the biological experiment. In addition, their respective specifity for alkali metal and alkaline earth metal ion binding would be interesting to know since this might also contribute to the observed L-selectin affinity. Such specific ion binding can be determined via NMR studies.[298] In order to proof the suggested conformational changes of dendritic polyanions depending on the size, charge density, and the environment further small angle X-ray scattering could be performed.[299] These data

can reveal the presence of complexed ions within the scaffold as well as the density of the interior dPG scaffold and hence contribute to a more in-depth understanding of the electrostatic binding of polyanions to biomolecules such as selectins.

6 Kurzzusammenfassung/ Short Summary

6.1 Kurzzusammenfassung

In der vorliegenden Arbeit wurde gezeigt, dass Glycerin-basierte Polymere mit linearer und verzweigter Struktur eine gute Alternative zu linearem Poly(ethylen glycol) (PEG) für die Beschichtung von Oberflächen darstellen. Mit diesen Polymeren konnte eine drastische Verringerung der nicht-spezifischen Adsorption von Proteinen und der Anlagerung von adhärenten Säugerzellen sowohl auf beschichteten Gold- als auch auf Glasoberflächen im Vergleich zu einer hydrophoben Kontrolloberfläche nachgewiesen werden. Weiterhin konnte gezeigt werden, dass sich im Vergleich zu einer nicht beschichteten Kontrolloberfläche weitaus weniger Bakterien auf diesen Polymer-modifizierten Oberflächen ansiedeln. Glycerin-basierte Polymere als Beschichtungsmaterial für biokompatible Oberflächen bieten im Vergleich zu PEG die Möglichkeit einer weiteren Derivatisierung der OH-Gruppen mit Liganden für die gezielte Wechselwirkung mit Biomolekülen. Solche Oberflächen sind insbesondere im Bereich Bioanalytik gefragte Materialien, da sie durch den Protein-resistenten Untergrund ein verbessertes Signal/Rausch Verhältnis aufweisen. Des Weiteren konnte mittels thermosensibler Glycerin-basierter Polymere gezeigt werden, dass die Protein- und Zellresistenten Eigenschaften dieser Polymere auf Oberflächen Temperatur gesteuert und reversibel „ein- und ausgeschaltet“ werden können. Solche schaltbaren Oberflächen haben insbesondere im Bereich Tissue Engineering hohes Potential als biokompatible 2D- oder 3D-Matrix.

Im zweiten Teil dieser Arbeit wurden hochfunktionalisierte Polyanionen auf Basis von dendritischem Polyglycerin hergestellt und hinsichtlich ihres anti-inflammatorischen Potentials via Selektinbindung untersucht. Es konnte gezeigt werden, dass dendritisches Polyglycerinsulfat (dPGS) mit hoher Affinität (IC_{50} Werte im nanomolaren Bereich) sowohl an L- als auch an P-Selektin bindet, jedoch nicht an E-Selektin. Eine in vitro Struktur-Aktivitäts-Studie wies auf, dass die Bindung von dPGS an L-Selektin sowohl mit dem Grad der Sulfatierung als auch mit der Partikelgröße zunimmt. Dabei bewirkt die chemische Natur der multivalent präsentierten anionischen Gruppen auf dem dendritischen Polyglycerin eine Steigerung der Affinität in der Reihenfolge Carboxylat < Phosphat < Phosphonat ≈ Sulfonat < Bisphosphonat < Sulfat. Diese Reihung entspricht tendenziell einer zunehmenden Säurestärke der anionischen Gruppe, während die direkte Korrelation der L-Selektin Bindung mit Partikelgröße und Zetapotential sowie deren Interpretation weiterer detaillierter Studien der Mikrostruktur der Polyanionen unter den Bedingungen des biologischen Experiments erfordern.

Mittels dieser in vitro und weiterer in vivo Studien konnte gezeigt werden, dass dPGS als vollsynthetisches Heparinanalogon ein viel versprechendes polymeres Therapeutikum für entzündliche Erkrankungen und daher ein geeigneter Kandidat für eine klinische Studie ist.

6.2 Short Summary

Within this thesis glycerol based polymers of linear and branched architecture were synthesized as poly(ethylene glycol) (PEG) equivalents and subsequently immobilized on surfaces. Such polymer modified gold and glass surfaces revealed a significant reduction of non-specific protein adsorption and mammalian cell adhesion as compared to a hydrophobic or non-coated control surface. Furthermore, reduced bacterial cell adhesion (> 99%) was observed on polymer coated glass as compared to bare glass. Glycerol based polymers in contrast to PEG as coating materials for biocompatible surfaces offer the possibility for further derivatisation of the multiple hydroxyl groups with ligands which induce specific interaction with biomolecules. These surfaces are highly demanded in particular in the field of bioanalytics because they reveal an enhanced signal to noise ratio due to the nonfouling background. Moreover, thermoresponsive glycerol based polymers for surface modification have been developed and it was shown by such modified surfaces that non-specific protein adsorption and cell adhesion can reversibly be triggered by temperature as an external stimulus. Such switchable surfaces are of specific interest in the field of tissue engineering as biocompatible 2D or 3D matrix.

Within the second part of this thesis highly functionalized polyanions based o dendritic polyglycerol were synthesized and evaluated for their anti-inflammatory potential via selectin binding. It was demonstrated that dendritic polyglycerol sulfate (dPGS) has high avidity for L- and P-selectin (IC_{50} values in the nanomolar range), but not for E-selectin. An in vitro structure-activity relationship revealed that dPGS binding to L-selectin increases with the degree of sulfation and the particle size. The nature of the multivalently presented anionic groups imparts L-selectin avidity and increases in the order carboxylate < phosphate < phosphonate ≈ sulfonate < bisphosphonate < sulfate. This ranking by trend reflects the increased acidity of the anionic groups. However, a straight correlation of L-selectin binding with particle size and effective charge as well as its interpretation requires further in-depth studies on the microstructure of the polyanions under the conditions of the biological assay.

These in vitro and further in vivo studies suggest that dPGS as a fully synthetic heparin analogue is a promising polymeric therapeutic for diseases related to inflammation and hence is a strong candidate for a clinical trial.

7 Appendix

In this section a few physical measurements will be introduced which were applied within this thesis as essential tools for evaluation of protein resistant surfaces and characterization of dendritic polyanions.

7.1 Surface plasmon resonance (SPR) spectroscopy[300]

In general, light is partially reflected when passing through an interface composed of a material of high refractive index and one with a lower refractive index as, for instance, at the glass/water interface. Thereby, total internal reflection is observed when the angle of incidence θ of the light beam is large enough. In a typical SPR spectroscopy experiment the top of the glass side, which is in contact with the fluid, aqueous medium, is coated with a thin semitransparent gold (~ 50 nm) layer (Fig. 32). Such a thin noble metallic film reduces the intensity of the totally reflected light. The incident angle at which a minimum of totally reflected light is observed is termed the surface plasmon resonance angle θ_{SPR}.

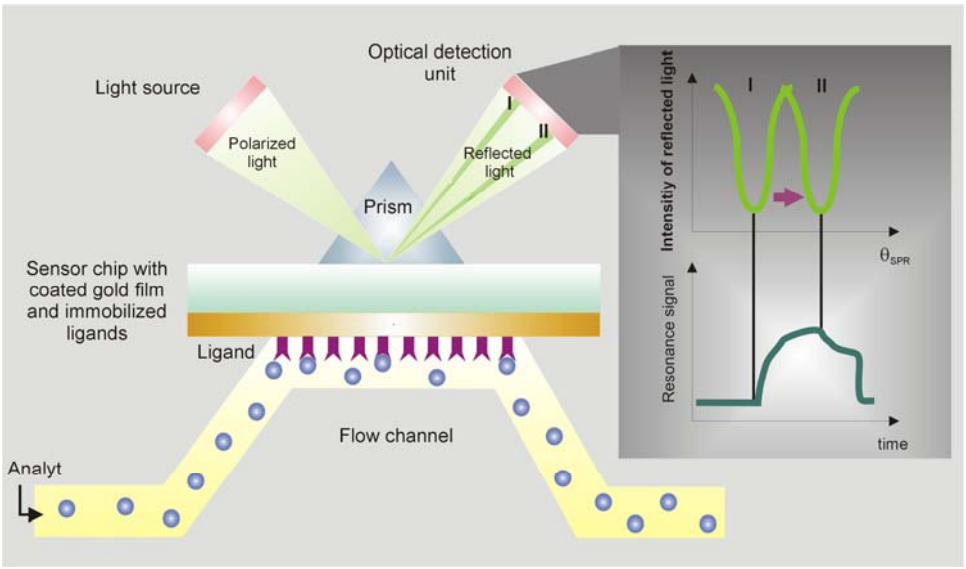


Figure 32. Illustration of a typicall SPR-spectroscopy set up.

The reduction of reflected light is attributed to the interference of the incident light with the mobile electrons at the metal surface which results in oscillation of the electrons. Such electrons are called surface plasmons. At θ_{SPR} the wave vector of the incident light matches exactly the wavelength of the surface plasmons and therefore sets them in resonance. This is associated with the generation of a decaying electrical field which extends for approximately 300 nm from the metal surface into the aqueous medium.

Due to this evanescent electrical field at the metallic/liquid interface the resonance frequency and thus θ_{SPR} is strongly dependent on the refractive index at this interface. Therefore, changes in the refractive index close to the metal surface result in small changes of θ_{SPR}. This high sensitivity of θ_{SPR} on the refractive index next to the metal surface is applied in SPR spectroscopy in order to detect label-free binding events in biology and biochemistry in real time with high accuracy. BIAcore™ for instance provides devices for SPR spectroscopy with integrated microfluidic cartridge, which includes the actual flow chamber, high precision pumps, automated sample injection tools, and a sensitive photo-detector array for the detection of shifts in θ_{SPR} as well as software for easy data processing and evaluation.[301]

In a typical SPR experiment the metallic surface is coated with a 2D or 3D monolayer which presents the immobilized ligand of choice. The free analyte is then passed over the surface within the microfluidic flow chamber. In the case of a binding event of the analyte to the ligand on the surface, a change in the refractive index close to the metal surface occurs which is detected via a shift in θ_{SPR}. Commonly shifts in θ_{SPR} are translated into response units (RU) and recorded as such, whereby 1 RU equals a change in θ_{SPR} of 0.0001°. Besides the detection and quantification of biological binding events SPR spectroscopy experiments offer access to thermodynamic data such as binding enthalpy as well as to kinetic data such as dissociation and rate constants while only very small quantities of analyte and ligands are required. However, in most cases a reference is needed in order to eliminate artefacts and non-specific interactions of analyte and ligand. Subtraction of the reference curve from the sample curve then reveals the actual biding event. In addition, the SPR angle is highly dependent on temperature which requires a constant and precisely controlled temperature setting during the experiment.

In addition to biological ligands immobilized on the chip the actual sensor chip surface can also be prepared by self-assembled monolayer (SAM) formation of thiol or disulfide comprising molecules on a blank gold coated sensor chip surface. SAM coated sensor chips are particularly valuable surfaces in the field of material's science for the investigation of new materials in contact with biofluids such as the evaluation of protein resistant surfaces.

7.2 Dynamic light scattering (DLS)[302]

According to Rayleigh scattering a beam of light is elastically and uniformly dispersed into all directions by small, spherical particles (< 60 nm) such as macromolecules in solution.[303] Because macromolecules in solution are constantly moving (Brownian motion), the waves of scattered light from neighbouring particles interfere in a constructive and destructive way and therefore result in intensity fluctuations. When monochromatic and coherent laser light is applied, these small fluctuations can be observed in a time resolved way and thus offer access to the diffusion constant D of the particles at constant temperature. Via the Stokes-Einstein (**2**) equation the hydrodynamic diameter d_h of the particles can be calculated when the diffusion constant D and the viscosity η of the applied solvent are known.

Stokes Einstein equation: $$D = \frac{k_B T}{3\pi\eta d_h} \qquad (\mathbf{2})$$

with k_B = Boltzmann constant, T = Temperature,
η = viscosity, d_h = hydrodynamic diameter

Under Rayleigh scattering conditions the intensity I of the scattered light is proportional to the sixth power of the particle diameter ($I \sim d^6$). The hydrodynamic size distribution via DLS is always obtained as a size distribution by intensity, which can mathematically be converted into size distribution by number or volume. In general, size by intensity is always larger than size by volume and number, but often overestimates the presence of larger particles in the sample, e.g., in case of partial aggregation, since scattering intensity is proportional to the sixth power of the particle diameter. Therefore, the size distribution by intensity in addition to size distribution by number or volume often helps to detect and identify the presence of small amounts of aggregates within the sample.

In order to minimize aggregation phenomena in the case of polyelectrolyte samples, measurements under high ionic strength are commonly recommended for an effective screening of the charges by ions which results in a lower degree of electrostatic and hydrogen bonding particle interactions.

7.3 Zeta (ζ-) potential measurement[303, 304]

A charged particle in solution is surrounded by an electrical double layer made up of ions due to coulomb forces. The electrical double layer consists of an inner tightly bound layer of ions of opposite charge to the particles' charge which is termed the Stern layer. The Stern layer is surrounded by a more loosely bound diffuse layer of ions. The built-up of an electrical double layer around a negatively charged particle is schematically illustrated in Figure 33 and the corresponding potentials as a function of the distance from the surface of the particle are indicated. Within the outer, diffuse layer exists a gradient of ions which approaches the bulk ion distribution in the solution at its outer edge. The diffuse layer has a boundary at a certain distance from the surface of the particle at which ions are not moving together with the particle anymore when under shear stress. As a result, this boundary is termed the surface of hydrodynamic shear and the corresponding, electrical potential at this boundary is defined as the zeta potential. Zeta potential measurements are electrokinetic measurements which are performed by applying an electrical potential to a conducting liquid containing the charged particles. The charged particles then start to move in the direction of the oppositely charged electrode while the loosely bound ions beyond the hydrodynamic shear boundary are stripped off by the shear stress. The velocity of the charged particles is dependent on the viscosity and dielectric constant of the liquid medium, the zeta potential of the particles, and the strength of the electrical field applied.

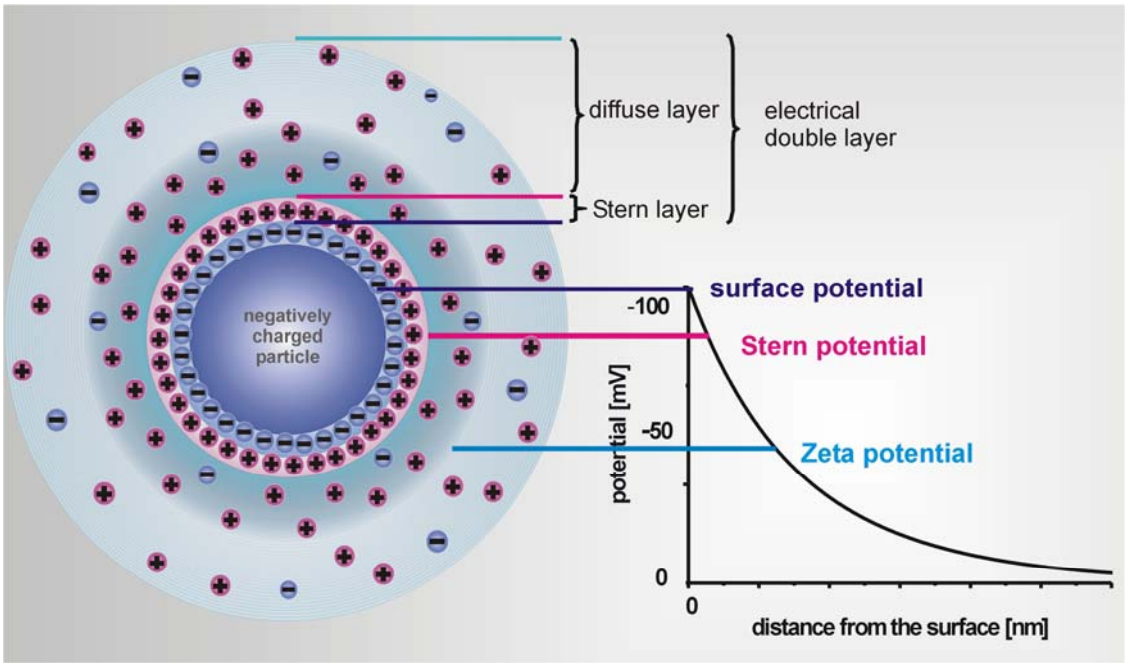


Figure 33. Schematic illustration of the electrical double layer with indicated surface-, Stern-, and zeta potential.

Under equilibrium the particles move with constant velocity and this electrokinetic mobility of the particles is measured in a typical zeta potential measurement by the scattering of laser light at the moving particles. Combination of the scattered light with the reference beam yields a fluctuating intensity signal with the result that the observed rate of fluctuation is proportional to the velocity of the particles. The measured electrokinetic mobility U_E can then be transformed into the zeta potential of the particles by means of the Henry equation (**3**).

$$\text{Henry equation:} \qquad U_E = \frac{2\varepsilon\xi f(\kappa a)}{3\eta} \qquad (\mathbf{3})$$

with η = viscosity of the medium
ε = dielectric constant of the medium
ζ = Zeta potential
$f(\kappa a)$ = Henry function, depending on the particle radius a and the thickness of the electric double layer $1/\kappa$.

For the Henry function two approximations are commonly used. The first is termed the Smoluchowski approximation ($f(\kappa a) = 1.5$) which is valid for large particles and high ionic strength media. The other is called the Hückel approximation ($f(\kappa a) = 1$) and is applied for small particles under low ionic strength conditions. In general, the zeta potential is strongly dependent on the applied electrical field, temperature, the ionic strength, and, most importantly, on the pH of the solution or dispersion and represents the effective charge of the particles under the applied conditions.

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9 Patent Applications, Publications and Conference Contributions

Patent Applications

1) Rainer Haag, Monika Wyszogrodzka, Marie Weinhart, *Production of linear, methylated polyglycerol derivatives for functionalization of surfaces in medical applications.* Ger. Offen. **2007**, 7pp. DE102006027125.

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3) Marie Weinhart, Ingo Grunwald, Monika Wyszogrodzka, Linda Gaetjen, Andreas Hartwig, Rainer Haag; *Linear Poly(methyl glycerol) and Linear Polyglycerol as Potent Protein and Cell Resistant Alternatives to Poly(ethylene glycol),* Chem. Asian J. **2010**, *5*, 1992-2000.

4) Adam R. Abate, Julian Thiele, Marie Weinhart, David A. Weitz; *Patterning microfluidic device wettability using flow confinement,* Lab on a Chip **2010**, *10*, 1774-1776.

5) Marcus Weber, Alexander Bujotzek, Karsten Andrae, Marie Weinhart, Rainer Haag; *Computational entropy estimation of linear polyether modified surfaces and correlation with protein resistant properties of such surfaces,* Molecular Simulation **2011**, *37,* 899-906.

6) Jens Dernedde, Alexandra Rausch, Marie Weinhart,Sven Enders, Rudolf Tauber,Kai Licha, Michael Schirner, Ulrich Zuegel, Arne von Bonin, Rainer Haag; *Dendritic polyglycerol sulfates as multivalent inhibitors of inflammation*, Proceedings of the National Academy of Sciences of the United States of America **2010**, *107*, 19679-19684.

7) Marie Weinhart, Tobias Becherer, Rainer Haag; *Switchable, biocompatible surfaces based on glycerol copolymers*, Chemical Communications **2011**, *47*, 1553-1555.

8) Marie Weinhart, Tobias Becherer, Nicolai Schnurbusch, Karin Schwibbert, Hans-Jörg Kunte, Rainer Haag, *Linear and Hyperbranched Polyglycerol Derivatives as Excellent Bioinert Coating Materials*, Advanced Engineering Materials **2011**, *13*, B501–B510.

9) Marie Weinhart, Dominic Gröger, Sven Enders, Sebastian B. Riese, Jens Dernedde, Rajesh K. Kainthan, Donald E. Brooks, Rainer Haag, *The Role of Dimension in Multivalent Binding Events: Structure-Activity Relationship of Dendritic Polyglycerol Sulfate Binding to L-selectin in Correlation with Size and Surface Charge Density*, Macromolecular Bioscience **2011**, *11*, 1088–1098.

10) Marie Weinhart, Dominic Gröger, Sven Enders, Jens Dernedde, Rainer Haag, *Synthesis of Dendritic Polyglycerol Anions and Their Efficiency Toward L-Selectin Inhibition*, Biomacromolecules **2011**, *12*, 2502-2511.

11) Gesine Gunkel, Marie Weinhart, Tobias Becherer, Rainer Haag, Wilhelm T. S. Huck, *Effect of Polymer Brush Architecture on Antibiofouling Properties*, Biomacromlecules **2011**, *12*, 4169-4172.

12) Kai Licha, Pia Welker, Marie Weinhart, Nicole Wegner, Sylvia Kern, Stefanie Reichert, Ines Gemeinhardt, Carmen Weissbach, Bernd Ebert, Rainer Haag, Michael Schirner, *Fluorescence Imaging with Multifunctional Polyglycerol Sulfates: Novel Polymeric near-IR Probes Targeting Inflammation*, Bioconjugate Chem. **2011**, *22*, 2453-2460.

Poster presentations

1) 1st European Chemistry Congress, Budapest, Hungary (27-31 August 2006)
   Poster: New Materials for Protein Resistant Surfaces;
   Marie Weinhart, Monika Wyszogrodzka, Conrad Siegers, Rainer Haag

2) Frontiers in Medicinal Chemistry, Berlin, Germany (18-21 March 2007)
   Poster: Dendritic Polyglycerol Sulfates as Highly Potent Selectin Inhibitors;
   Marie Weinhart, Jens Dernedde, Sven Enders, Rainer Haag

3) IDS:5 – International Dendrimer Symposium 5, Toulouse, France (28 August - 1 September 2007)
   Poster: Dendritic Polyglycerol Sulfates as Highly Potent Selectin Inhibitors;
   Marie Weinhart, Jens Dernedde, Sven Enders, Rainer Haag

4) REACT 2007, 3rd International Symposium on "Reactive Polymers in Inhomogeneous Systems, in Melts, and at Interfaces", Dresden, Germany (23 - 26 September 2007)
   Poster: Dendritic Polyglycerol Sulfates as Highly Potent Selectin Inhibitors;
   Marie Weinhart, Jens Dernedde, Sven Enders, Rainer Haag

5) BIOTECHNICA 2007, Hannover, Germany (9-11 October 2007)
   Poster: Dendritic Polyglycerol Sulfates as Highly Potent Selectin Inhibitors;
   Marie Weinhart, Jens Dernedde, Sven Enders, Rainer Haag

6) International Symposium Bioactive Surfaces 2010, Golm, Germany (20 May 2010)
   Poster: Linear and Hyperbranched Polyglycerol Derivatives as Excellent Biocompatible Glass Coating Materials
   Marie Weinhart, Tobias Becherer, Rainer Haag

7) Polydays 2010, Berlin, Germany (3-5 October 2010)
   Poster: Glycerol Based Polymers for Biocompatible Surface Modifications;
   Marie Weinhart, Tobias Becherer, Rainer Haag

Oral presentations

1) Bionnale Speed Lecture Award 2009, Berlin, Germany (29 April 2009)
   Oral presentation: Dendritische Polyglycerin Sulfate (dPGS) als potente Selektin-inhibitoren bei entzündlichen Erkrankungen
   Marie Weinhart, Jens Dernedde, Sven Enders, Rainer Haag

2) Trilateral Symposium on NanoBio Integration, Berlin, Germany (30 September – 3 October)
   Glycerol Based Polymers for Biocompatible Surfaces
   Marie Weinhart, Rainer Haag

3) 5. Senftenberger Innovationsforum Multiparameteranalytik, Senftenberg, Germany (10 March 2011)
   Glycerol basierte Polymere für bioinerte, schaltbare und biofunktionale Oberflächen
   Marie Weinhart, Rainer Haag

10 Curriculum Vitae

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