On the Development of Mucin-based Biomaterial Coatings

TOMAS SANDBERG
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Abstract

Owing to their key role in mucosal functioning as surface barriers with biospecific interaction potentials, the mucins are interesting candidates for use as surface modifiers in biomaterials applications.

In this work, “mild” fractionation procedures were used to prepare mucins of bovine (BSM), porcine (PGM), and human (MG1) origin. Biophysicalchemical analysis showed the prepared mucins to differ in size, charge, conformation, and composition. In turn, these factors were shown to govern mucin adsorption on hydrophilic and hydrophobic model surfaces.

To enable for detailed coating analysis, methods for the qualitative and quantitative analysis of mucin-based coatings were developed. Of particular interest, a method for the determination of the fraction of surface-exposed, presumed bioactive proteins in a complex mucin coating was described.

It was shown, using microscopy and activation assays, that mucin precoating effectively suppresses the neutrophil response towards a polymeric model biomaterial. Under optimal coating conditions, all mucins performed equally well, thus indicating them to be functionally similar. Coating analysis suggested that efficient mucin surface-shielding is critical for good mucin coating performance.

Following a study on the complexation of albumin with preadsorbed mucin, we investigated the effect of mucin precoating on the conformation and neutrophil-activating properties of adsorbed host proteins. We found that mucin precoating greatly reduces the strong immune-response normally caused by adsorbed proinflammatory proteins (IgG and sIgA). Detailed coating analysis revealed that the fraction of surface-exposed protein in the mucin-protein composite influences the neutrophil response. Unexpectedly low neutrophil activation for composites containing near-monolayer concentrations of exposed IgG, suggested IgG to act synergistically with mucin on the surface. Conformational analysis supported this by showing that a preadsorbed mucin layer could stabilize adsorbed IgG through complexation. Our findings link well to the complex in vivo situation and suggest that functional mucosal mimics can be created in situ for improved biomaterials performance.

Keywords: Mucin, Biomaterial, Surface-exposed protein, XPS, Neutrophil, Cell morphology, SEM, QCM-D, Viscoelasticity, Protein-stabilization, HNL, Coating, MG1, BSM, PGM, SEC-MALS-RI, Mucin quantification, Protein adsorption, Ellipsometry

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Dedications

_Till mamma_,

_För alla läxkvällar vid köksbordet._
_För tålamodet att lyssna i svåra stunder._
_För tron på den lilla tjuriga killen i Adidas-skorna._

_Till Xia, Klara, Krister och pappa_,

_För tålmodigt väntande._
List of papers

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


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**My contribution to the papers:**

Paper I-IV, VI: I developed the main ideas behind these works and did the main part of the experimental planning and the practical work. I wrote all the papers and in some cases acted corresponding author (III, IV).

Paper V: I developed the main ideas behind this work and contributed to the writing of the paper.

**Work not included in this thesis:**


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# Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>Molar refractivity</td>
</tr>
<tr>
<td>(A_2)</td>
<td>Second virial coefficient</td>
</tr>
<tr>
<td>ABS</td>
<td>Acetate-buffered saline</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCIP-NBT</td>
<td>5-bromo-4-chloro-3-indolyl phosphate- Nitro blue tetrazolium</td>
</tr>
<tr>
<td>Biotin</td>
<td>5-[(3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl]pentanoic acid</td>
</tr>
<tr>
<td>BSM</td>
<td>Bovine submaxillary gland mucin</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl trimethylammonium bromide</td>
</tr>
<tr>
<td>D</td>
<td>Dissipation factor (film)</td>
</tr>
<tr>
<td>(d_{\text{eff}})</td>
<td>Effective thickness (film)</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELLA</td>
<td>Enzyme-linked lectin assay</td>
</tr>
<tr>
<td>f</td>
<td>Frequency</td>
</tr>
<tr>
<td>Fbg</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HNL</td>
<td>Human neutrophil lipocalin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse-radish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>HWS</td>
<td>Human whole saliva</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>(I_p)</td>
<td>Protein (N1s) peak intensity (XPS)</td>
</tr>
<tr>
<td>Luminol</td>
<td>5-amino-2,3-dihydro-1,4-phthalazinedione</td>
</tr>
<tr>
<td>MALS</td>
<td>Multiangle light scattering</td>
</tr>
<tr>
<td>MG1</td>
<td>High molar mass human salivary mucin</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>(M_w)</td>
<td>Molar mass, molecular weight</td>
</tr>
<tr>
<td>(N (=n+ik))</td>
<td>Complex refractive index</td>
</tr>
</tbody>
</table>
Effective refractive index (film)
Amine-functionalized PTFE
Polyacrylamide gel electrophoresis
Phosphate buffer
Polybisphenol A carbonate
Phosphate-buffered saline
Polydimethyl siloxane (silicone)
Polyethylene
Polyethylene terephthalate
Piperazine gastric mucin
Piperazine-buffered saline
Polymorphonuclear granulocyte, a.k.a. neutrophils
4-Nitrophenyl phosphate
Polypropylene
Proline
Polystyrene
Polytetrafluoroethylene
Polyvinylchloride
Quartz crystal microbalance with dissipation monitoring
Hydrodynamic radius
Refractive index detector
Reactive oxygen species
Root-mean-square radius
Sodium dodecylsulfate
Size-exclusion chromatography
Scanning electron microscopy
Serine
Secretory immunoglobulin A
Static light scattering
Tris-buffered saline
Tris-EDTA buffer
Threonine
X-ray photoelectron spectroscopy
Ångström; 10^{-10} m
3,3',5,5'-Tetramethylbenzidine
Medium viscosity
Shear viscosity (film)
Electron inelastic mean free path
Shear elastic modulus (film)
Characteristic relaxation time (film)
Introduction

Biomaterials

From the most general point of view, biomaterials are materials intended for contact with living tissue. Following this broad definition, the term “biomaterial” includes materials used in *in vivo/ex vivo* (e.g. heart valves and oxygenator membranes) as well as in medical (e.g. cell culturing) and non-medical (e.g. biotechnological process equipment) *in vitro* applications. Classically and still, there is a focus on the *in vivo* applications. Limiting this thesis to such applications, biomaterials are essentially materials used to, totally or partially, sense, support, or replace lost body function caused by injuries or diseases [1]. The ultimate goal of medical biomaterials research is the creation of materials that can perform optimally without causing negative side-effects. Specifically, the biomaterial should be biocompatible, i.e. have the ability to perform with an appropriate host response in a specific application [2].

Increased knowledge in the clinical field has deepened our understanding of the mechanisms ruling biomaterials performance *in vivo* and in turn accelerated the development of new biomaterials. A simple database search gives at hand that the biomaterials field has grown exponentially as a research discipline during the last 18 years (Fig. 1).
Classes of materials used in the biomaterials field

Different medical applications put different demands on the material characteristics. For example, the required tensile strength of bone implants differs considerably from that required for implants used only in contact with soft tissue. Although the term “biomaterial” makes no direct distinction between materials from artificial and natural sources, the artificial materials are in a majority among the used bulk materials. This predominance could be ascribed to their good mechanical properties, good availability and relatively controllable characteristics. Historically, there has been a strong belief in fully translating natural systems into artificial counterparts. However, with the emerging field of tissue engineering, today’s trend is towards natural materials. That is, these materials are already highly adapted to the biological environment and do therefore often induce lower immune responses than do artificial materials. The ultimate natural materials are the autografts, which include cells and tissues originating from another site of the same patient. Although autografts represent highly “ideal” materials, there are several situations when materials are needed from external sources such as other humans (allografts), or animals (xenografts). However, although these materials are apparently similar to the autografts, they often suffer from “non-self” reactions, which eventually lead to their rejection. The great majority of natural materials are in fact not whole transplants as being described above, but single substances, extracted from human, animal or plant sources. Particularly, as will be discussed in a coming section, extracted natural sub-

Figure 1. “Biomaterial” and “Medical implant” as search terms in SciFinder™, years 1990-2007.
stances are favorably used as surface modifiers to improve the biocompatibility of artificial materials. In that way, the good mechanical properties of the artificial materials could be utilized, although their surfaces often are immuno-reactive.

Based on their biological performances, materials could be classified as either bioinert or bioactive [3]. A bioinert material shows low level of tissue integration (e.g. biosensor applications). In contrast, a bioactive material interacts readily with the surrounding tissues and show high level of integration (e.g. bone implant applications). One interesting subclass of the bioactive materials is the resorbable materials. These materials provide temporary mechanical support and stabilization before being degraded into harmless byproducts which are absorbed by the body (e.g. sutures).

Biomaterials are normally divided into polymers, metals, ceramics and natural materials. Combinations of two or more materials are referred to as composites. Below follows short descriptions of these material classes. Table 1 presents examples of each material class together with some specific applications.

**Polymers** [4] are long-chain, high molar mass macromolecules built from a large number of smaller units (monomers) of organic origin. Their physicochemical properties depend mainly on their chemical composition, molar mass distribution and degree of cross-linking. Polymers may be of synthetic or natural origin; due to their good mechanical properties, the synthetic polymers dominate as bulk materials. However, today’s trend is towards polymers, which link more closely to the natural environment. One important subclass in this sense is the degradable/resorbable synthetic polymers [5]. Another interesting subclass is the so-called “smart polymers”, i.e. polymeric materials that respond to stimuli in the body and adapt their function to the local environment [6]. The polymers are used in many different applications such as joint prostheses, intraocular lenses, vascular grafts, breast implants and pacemakers.

**Metals** [7] are relatively nondeformable materials which are used in hard tissue applications. These include total hip joints and fracture fixation devices. The most commonly used metals are titanium, stainless steel and chromium-cobalt alloys. Titanium and its alloys have become particularly popular choices since they have suitable elastic moduli and are relatively resistant to corrosion.

**Ceramics (sometimes bioceramics)** [8] (including the carbons, glasses and glass-ceramics) is a category of materials based on inorganic/non-metallic substances. The ceramics are usually divided into bioinert, resorbable, bioac-
tive and porous types. In resemblance with the metals, the ceramics are hard and insoluble in water. However, they show considerably lower mechanical strength and do often suffer from fatigue failure. These drawbacks are to some degree balanced by their high biocompatibility. Applications include replacements for teeth, knee and hips, and bone reconstructions.

**Natural materials** [9] could originate from human, animal, or plant sources. Focusing on materials for bulk use, many of the natural materials are biopolymers that normally possess structural and/or connective functions. These include proteins such as collagen and polysaccharides such as cellulose. Compared to the synthetic polymers the biopolymers are relatively unstable, and therefore mostly used in resorbable/degradable applications. Although, originating from natural sources, some of the biopolymers are quite immunogenic. New technologies, including the recombinant protein technology, have improved this situation by enabling for the preparation of highly contaminant-free natural materials (modified natural materials). Applications using natural materials include hemodialyzer and corneal bandage.

**Composites** [10] are combined (on a macroscopic level) materials based on two or more of the above material categories, commonly polymers and ceramics. The composites account for the fact that many biomaterial applications demand for highly complex material characteristics, not necessarily covered by one single material. Good examples of composites are calcium phosphate/aramid fibers in bone cement applications, and polyethylene/hydroxyapatite particles in bone substitute applications.
Table 1: Examples of materials used in the biomaterials field, together with some specific applications.¹

<table>
<thead>
<tr>
<th>Material</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metals</strong></td>
<td></td>
</tr>
<tr>
<td>Titanium</td>
<td>Joint replacements, dental applications</td>
</tr>
<tr>
<td>Stainless steel</td>
<td>Joint replacements, bone fracture fixation</td>
</tr>
<tr>
<td><strong>Ceramics</strong></td>
<td></td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>Bone defect repair</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>Dental implant for tooth fixation</td>
</tr>
<tr>
<td><strong>Polymers</strong></td>
<td></td>
</tr>
<tr>
<td>Silicone</td>
<td>Catheter, heart-lung machine</td>
</tr>
<tr>
<td>Teflon</td>
<td>Blood vessel prosthesis, catheter, artificial tendon and ligament</td>
</tr>
<tr>
<td>Dacron</td>
<td>Blood vessel prosthesis, artificial tendon and ligament</td>
</tr>
<tr>
<td>Polyethylene</td>
<td>Joint replacements</td>
</tr>
<tr>
<td>Poly methyl methacrylate</td>
<td>Intraocular lens, bone cement</td>
</tr>
<tr>
<td><strong>Natural materials</strong></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>Corneal bandage</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Hemodialyzer</td>
</tr>
<tr>
<td><strong>Composites</strong></td>
<td></td>
</tr>
<tr>
<td>Silicone-collagen</td>
<td>Skin repair template</td>
</tr>
</tbody>
</table>

¹ Data from reference [1].

**Host-biomaterial interactions**

**Initial phase: Protein-mediated host response**

Immediately upon implantation, the biomaterial (implant) meets and interacts with soluble host components present in direct proximity to the implant [11,12]. These originate mainly from blood and include mobile species such as water, ions, lipids, carbohydrates, amino acids and proteins. Within seconds to minutes after implantation, a complex surface layer starts to form on the implant surface (Fig 2).
Figure 2. Schematic representation of the interaction between soluble host components and the implant surface following implantation.

The character of the implant surface governs the interaction behavior of the arriving host components. As will be further discussed, both physical and chemical factors are of concern. Physical factors include hardness and topography, and chemical factors include hydrophobicity and the presence of specific functional groups.

It is well established that the layer of host proteins initially formed on the implant surface governs the acute host response [11-14], which in turn determines the long-term outcome of the implantation [11,12]. Originating mainly from blood, many of the first-arriving host proteins are part of or have an impact on the defense systems involved in body homestasis, primarily the host coagulatory [15,16] and complement [17,18] systems. Proteins of particular importance are the immunoglobulins (mainly IgG) [17-19], fibrinogen [15,16] and surface-active complement components (mainly C3) [17,18].

**Fundamentals of protein adsorption**

As noted from the above, the initial adsorption of host proteins to the implant surface is a common theme underlying the host response. It follows that, knowledge about how to control host protein adsorption to implant surfaces, is important for the development of biocompatible materials.

Fundamental theory states that regardless of mechanism and kinetics, protein adsorption can occur only if Gibbs energy ($G$) of the total system decreases:

$$
\Delta_{ads} G = \Delta_{ads} H - T \Delta_{ads} S < 0
$$

(1);

where $H$, $S$, and $T$ refer to enthalpy, entropy, and absolute temperature, respectively, and $\Delta_{ads}$ relates to the change in state functions occurring on adsorption. In other words, if $\Delta_{ads} G$ is negative (release of energy), the process...
is spontaneous and there is a driving force for adsorption. Vice versa, if the \( \Delta_{ads} G \) is positive, the process is non-spontaneous and there is no driving force for adsorption. There are several factors that influence \( \Delta_{ads} G \), and in turn the surface interaction of the protein. According to the above, both physical and chemical factors are to be considered. The very same type of interactions that control protein conformation and stability, also govern the adsorption of proteins to artificial surfaces. These include electrostatic interactions (ion-ion, dipole-ion and dipole-dipole), hydrophobic interactions, dispersive interactions (van der Waals), and hydrogen bonding. The main processes underlying protein adsorption are considered to be 1) charge redistribution, 2) hydrophobic dehydration, and 3) structural rearrangements [20,21].

*Charge redistribution* relates to the fact that most surfaces and practically all proteins are charged, and interact with each other as well as with charged species of the surrounding liquid medium. Protein adsorption leads to a redistribution of the charges in the system, which from a thermodynamic point of view may be either favorable or unfavorable, all according to eq. 1. Often, we could find a good answer to whether adsorption is spontaneous or not by looking at the charge states of the protein and the surface, respectively. For example, if a protein and a surface are of opposite charge, they generally experience an attractive interaction. Since charge-charge interactions (sometimes simply referred to as electrostatic interactions) could act over relatively large distances, they are important in many adsorption processes. Factors influencing the strength of the electrostatic interactions include the pH and ionic strength of the surrounding medium. In systems of high ionic strength, such as in most biological systems, charged species are screened and the effect of electrostatic interaction reduced.

*Hydrophobic dehydration* is the process in which water-exposed hydrophobic regions increase their contact with other hydrophobic regions and as a consequence expel associated, ordered water. Naturally, most proteins bury their hydrophobic regions in the internal cavities of the protein, thereby minimizing contact of these regions with water. However, since considerable interaction with water often still occurs, it may be energetically favorable for the protein to adsorb to a hydrophobic surface and thereby reduce water contact even more. In fact, one common measure of the immuno-reactivity of a material is the hydrophobicity of its surface. That is, hydrophobic materials, in contrast to hydrophilic materials, are prone to induce protein dehydration, in turn leading to host protein adsorption and subsequent immuno-activation.

*Structural rearrangements* relates to the entropy term in equation 1. That is, many native proteins are arranged in highly ordered states, which are controlled by intramolecular interactions. Upon adsorption, the protein structure
may rearrange to account for the additional interaction with the surface. Generally such rearrangements lead to a gain in entropy and therefore are energetically favorable. As a rule of thumb, proteins of low structurally stability (“soft” proteins) adsorb more readily than do proteins of high structurally stability (“hard” proteins). Structural rearrangements are very important to protein adsorption, and could in fact even overcome effects of electrostatic repulsion.

From a biomaterials point of view, one should keep in mind that the adsorption of a protein is a dynamic process which also involves surface relaxation (surface-induced structural rearrangements) and surface exchange. Normally, several proteins adsorb simultaneously to the surface under highly competitive conditions. The so-called Vroman effect [22] relates to this behavior, i.e. smaller, mobile proteins dominate the surface at start but do become exchanged for larger, preferrably “softer”, proteins over time (Fig. 2). Size is also a factor of concern. Generally, due to more interaction points, the surface interaction of a large protein is stronger than that of a small protein of the same composition.

Secondary phase: Cellular host response

Over time (minutes to a few hours), the cellular host response starts [12]. First to arrive to the scene of implantation, are the platelets, which are highly abundant in blood and brought there passively from leaking blood vessels close to the implant site. Shortly after, the neutrophils arrive, migrating there actively in response to chemical factors originating from the implant site. The platelets and neutrophils are considered the main cellular mediators of acute host response. Upon arrival to the implant, these cells “sense” the implant surface and its surroundings for specific factors which could trigger them to respond. Such factors include adverse topographic features, surface-associated protein “markers” (opsonins) and injured tissue. If they do respond, a complicated series of reactions takes places, which involves cell adhesion, cell-cell contacts and cell-specific responses (Fig. 3). Thus, while the platelets start to form a clot (thrombus) on the surface [15] the neutrophils try to engulf and degrade the material (phagocytosis) [12].
Neutrophils as models of acute host response

The neutrophils, also known as polymorphonuclear leukocytes/granulocytes (PMNL or PMN), are the most abundant circulating white blood cells (leukocytes), constituting approximately half the leukocyte population. This cell type is an important member of the innate immune system and considered the first defense line against infectious agents, normally including exogenous cells, bacteria and viruses [12,19,23]. Initially, circulating neutrophils respond to injured tissue/foreign matter by adhering loosely to the vascular endothelium in a process referred to as “neutrophil rolling” [24]. Firm binding eventually stimulates the secretion of enzymes that degrade the endothelium wall and enable neutrophil passage. Outside the blood vessel, the neutrophils move towards the site of injury by following gradients of chemotactic factors (chemotaxis) [12]. Neutrophils are guided locally by opsonins, primarily complement factor C3b and immunoglobulin G’ (IgG) [12,17], which have “marked” the injured tissue/foreign material. As indicated above, the neutrophils function by degrading/engulfing the foreign material. This process involves secretion of granules (primary, secondary and tertiary) containing several proteases, lactoferrin, lysozyme, and myeloperoxidase, of which the latter catalyzes the production of reactive oxygen species (ROS) [25,26]. Diagnostic markers of primary and secondary granule releases are myeloperoxidase [27] and human neutrophil lipocalin [28], respectively. As the neutrophils produce ROS their consumption of oxygen increase, something referred to as the oxidative burst [29]. If the foreign material is too large in size, which most biomaterials are, the neutrophils cannot phagocytose, leading to a situation referred to as “frustrated phagocytosis” [12]. In this state, the neutrophils maximize their spreading, flatten and do their best to engulf the implant, all under the secretion of large amounts of granules (Fig. 4).
Although, inflammation is a complex event, involving a multitude of actors, the neutrophils are key players in this process and as such generally considered good models of biomaterial-induced inflammation [29].

**Figure 4.** Neutrophils undergoing “frustrated phagocytosis” on a polymeric surface (SEM; 6000x magnification).

**Final phase: Regeneration versus foreign body reaction**

In addition to the above events, the platelets and neutrophils secrete cytokines, which in turn attract monocytes to the implant. These eventually differentiate into macrophages, which in similarity with the neutrophils react to the foreign material by trying to destroy/engulf it [30]. Again a state of frustrated phagocytosis arises. If the macrophages are triggered continuously they may fuse to form foreign body giant cells, which maintain the inflammatory activities for long time, more or less leading to a chronic inflammation (foreign body reaction) [11,12].

In parallel with the inflammatory activities described above, several processes take place that intend to heal the wound [12]. These include the formation of highly vascularized connective tissue (granulation tissue), which over time transforms into collagen-rich fibrous tissue. In a normal wound, without inflammatory activities, the fibrous tissue transforms further to regenerate normal tissue (parenchymal tissue). However, when an inflammation is ongoing, the fibrous tissue formation continues and the implant may eventually become completely screened from the rest of the body by fibrous tissue (fibrous encapsulation) [12].
Clearly, the final outcome of the implantation, towards healing/implant integration by regeneration of wounded tissue or towards encapsulation and/or chronic inflammation, is a delicate balance between the inflammatory and healing processes. Additional factors not covered in this discussion but of great importance are systemic effects (toxicity, hypersensitivity, and thromboembolic complications) [31,32], tumorigenesis [33] and biomaterial-related bacterial infections [34].

Surface modification strategies

It is clear from the above that it is mainly the characteristics of the implant surface, which governs the acute and in turn long-term host response. Thus, one powerful strategy to improve biomaterial performance is to modify the surface of the material in such a way that it can facilitate more favorable host reactions [35-38]. This is of particular importance for the synthetic polymers, which normally have surface chemistries that provoke negative host reactions. We can discern two different modification objectives. One often-used objective is to create inert, non-fouling surface barriers, which can shield the surface from adsorbing host components [39]. Another, perhaps more logical alternative if long-term integration is considered, is to create modifications which could facilitate a controlled interaction between the surface and the invading host components [36,38,40].

Surface modification may be achieved by 1) chemically altering the existing surface (chemical modification), 2) physically covering the existing surface with a substance that have more favorable properties (physical modification), or 3) using a combination of 1) and 2). Below follows short descriptions of the main principles behind these modification strategies together with some examples.

Chemical modification [35] concerns the creation of new chemical groups on the surface through the covalent binding of one or more specific substances. For polymeric materials, this could be achieved by using a corona discharge plasma reactor, where the material is reacted with electrically activated substances in gas phase [41]. Another alternative is to react the surface with a suitable substance in solution [42]. Promising gas phase-modifications for use in ultra-low binding blood applications exist [43]. While the surface distribution of the created groups generally is hard to control using such random reaction mechanisms, more controlled ways of introducing desirable functionalities on a polymeric surface have been described [44]. Common problems with chemically modified materials include migration of grafted surface groups into the bulk material. The same mechanism, although inverted, underlies a promising strategy referred to as self-asssembling monolayer end group (SAME) modification [45].
Physical modification [35] relies on the physical enclosure (coating) of the bulk material by one or more specific substances. Chemical modifications do many times precede the physical modification, and the stability of physically adsorbed substances are many times improved by means of post-chemical modification, where the substance is covalently bound to the surface or cross-linked internally. Several substances have been studied over the years, originating from synthetic as well as natural sources. Classification could be made on physicochemical as well as biological basis, i.e. these substances may be polymeric, charged, neutral, hydrogel-forming, bioactive, bioinert and so forth. Since the target materials often are polymer-based and hydrophobic in nature, and since hydrophobic materials generally induce stronger immuno-responses than do hydrophilic surfaces, a common strategy is to make the surface more hydrophilic. Good examples of coating substances are poly(vinyl alcohol) (PVA) (synthetic polymer) [46], poly(vinyl pyrrolidone) (PVP) (synthetic polymer) [36], hyaluronan (natural polysaccharide) [47], gelatin (natural protein) [48], dextran (natural polysaccharide) [48], albumin (natural protein) [49] and heparin (natural carbohydrate) [49]. A very popular class of substances that could render the surfaces more hydrophilic as well as improve protein-resistance is the polyethyleneglycol (PEG) derivatives. Particularly, the so-called poloxamers, i.e. block-copolymers between polyethyleneoxide (PEO) and polypropyleneoxide (PPO), have been studied diligently [50-52].

Mucins: Natural surfactants with interesting coating potentials

Owing to their biological functions, the mucins are interesting candidates for biomaterial coating applications. Below follows an introduction to mucin biology and a short summary of past mucin studies in the surface science field.

Mucin biology

Basic structure

The mucins constitute a structurally diverse class of extracellular glycoproteins of generally high molar mass (0.2-20 MDa) [53,54]. Based on the presence or absence of a transmembrane domain-encoding part in their genes, mucins are divided into secreted and cell-associated variants. Shared structural features include the heavily glycosylated, hydrophilic “mucin domains” (tandem-repeat domains) and the nonglycosylated, more hydrophobic “protein domains” [53,54].
The mucin domains are normally located at the mid-part of the mucin peptide sequence and central to mucin structure and interaction behavior. They are rich in repeats of proline, and threonine and/or serine. Oligosaccharides of various composition and length (5-15 monomers) O-link to the serine and threonine residues. Mucin carbohydrate contents often exceed 80% of the total mass. The complex glycosylation strongly contributes to the large variability of the mucins. Most oligosaccharides are terminated by sialic acid (pKa ~2.6) and/or sulfate (pKa ~1), which make mucins negatively charged under most physiological conditions. The charged mucin domains are responsible for mucin intramolecular repulsion, which underlie their expanded, "bottle-brush" structure. In addition, by containing large numbers of hydrogen-bonding groups, the mucin domains confer hydrophilic properties to the mucins.

The protein domains are essentially nonglycosylated and flank the mucin domains towards the N- and C-terminal parts of the mucins. In addition, some mucins have one or more protein domains interspersed within the mucin domains. Structural motifs of relevance within the protein domains include the Cys subdomains and the D- and CK-like domains of the secreted mucins and the transmembrane, EGF-like and SEA-like domains of the cell-associated mucins. The D- and CK-like domains, and Cys subdomains are cysteine-rich (>10%) and take part in the intermolecular bonding between mucins. The transmembrane domains of cell-associated mucins link to the epithelial cell membrane. Generally, the proteins domains confer hydrophobic properties to the mucins, thereby being the underlying factor behind mucin self-association, mucin adsorption on hydrophobic surfaces and mucin interaction with other proteins (see below).

The secreted, gelforming mucins are assembled intracellularly before secretion [53]. In this process, disulfide bonds are initially formed between the C-terminal CK-like domains of two mucin monomers. The formed dimer is then glycosylated and sulphated, before being assembled into disulfide-bonded oligomers-multimers through the N-terminal D-domains. Figure 5 presents a schematic representation of a secreted, gelforming mucin.
Figure 5. Schematic representation of a secreted, gelforming mucin. N and C identify the amino and carboxyl termini of the core peptide, respectively. Major domains include: the mucin domains, which contain densely packed O-linked oligosaccharides, the D- and CK (Cystine knot)-like domains, which take place in mucin multimerization, and the Cys subdomains, which have unknown function. As illustrated by one of the insets, the carbohydrate side-chains contain sialic acid and sulfate groups, giving the mucins a net negative charge at neutral pH. More detailed theory on mucin structure is found in [53,54].

Expression
The mucins are found at the epithelial cell linings of all vertebrates and at the skin of amphibians and fish [53]. To this date, 19 human mucin gene products have been identified [55], of which 12 have been fully sequenced [53]. Expression sites include the respiratory, oculo-rhino-otolaryngeal, gastrointestinal and urogenital tracts. Mucin homologs have been described for mouse and rat, and mucins from bovine and porcine sources show large sequence similarities with human mucins. According to conventions the human mucin gene products are named MUC accompanied by an index (1-19). Notably, many of the smaller cell-associated mucins are not considered “true” mucins since they only share their mucin domains with other mucins [54]. Of the “true” mucins, we note MUC2, MUC5AC, MUC5B and MUC6 among the secreted gelforming mucins, and MUC7 among their non-gelforming counterparts. MUC1, MUC4 and MUC16 are notable members of the cell-associated mucins. While the cell-associated mucins are expressed on most epithelial cells the secreted mucins are only produced by specialized cell types (goblet cells and mucus gland cells) found to a varying degree in each tissue. References [56] and [57] present nice recent reviews of the structure and function of some important secreted and cell-associated mucins, respectively. Table 2 summarizes the tissue distribution of some common human mucins.
<table>
<thead>
<tr>
<th>Mucin</th>
<th>Tissue location (examples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secreted</td>
<td></td>
</tr>
<tr>
<td>MUC 2</td>
<td>Colon and small intestine (GC), salivary gland ducts, conjunctival Ep.</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>Bronchus (superficial GB, SMC), colon (GB), superficial stomach ep., endocervical ep., conjunctival ep.</td>
</tr>
<tr>
<td>MUC5B</td>
<td>Bronchus (SMC) salivary glands, gall bladder ep., colon (GB)</td>
</tr>
<tr>
<td>MUC6</td>
<td>Gastric ep. (mucus neck cells), small intestine (GB), colon, gall bladder ep., pancreas, endometrial ep.</td>
</tr>
<tr>
<td>Cell-associated</td>
<td></td>
</tr>
<tr>
<td>MUC1</td>
<td>Widely distributed among ep. cells, also present on fibroblasts</td>
</tr>
<tr>
<td>MUC4</td>
<td>Bronchus, colon, conjunctival ep, middle ear</td>
</tr>
</tbody>
</table>

1Data adapted from [53]. GC = goblet cells, SMC = submucosal mucus cells, and ep. = epithelium

**Solution conformation, self-association and gel formation**

Light scattering studies have revealed that mucins under dilute solution conditions exist as stiff random coils [58]. Their stiff, extended state is caused by intramolecular repulsion between peptide-linked GalNAc residues of the carbohydrate side-chains and adjacent amino acids in the core peptide [59]. Charge repulsion between neighbouring side-chains does also add to the stiffening [60]. The stiffening effect in the mucin domains extends to the nonglycosylated protein domains. Interestingly, it has been shown that only the very first residues in the carbohydrate side-chains are needed to maintain the chain-stiffening effect [58].

Due to their large sizes and extended conformations, the mucins start to overlap at relatively low concentrations in solution. Under physiological conditions, overlap concentrations for mucins normally range 2-4 mg/mL [61]. Overlapping leads to entanglement/aggregation/gelation of the mucins, which is manifested as an increased viscosity of the solution. The main contribution to self-association is nonspecific interaction between nonglycoslated, hydrophobic protein domains in the mucins [62,63]. Detailed studies on porcine gastric mucin at low pH have suggested that these hydrophobic interactions are preceded by conformational changes within the protein domains [62,64].

High ionic strength conditions generally collapse the mucin structure, due to reduced intramolecular repulsion, thereby preventing gel formation [65].
Mucin-protein interactions and mucus formation

Although purified mucins self-associate under high concentration conditions to form gels, it has been shown that such gels do not replicate the native mucus [66]. From this finding it was suggested that normal mucus formation depends on the action of other components in the secretion [66]. Indeed, other studies confirm that other components could have an impact on the mucin gelation process. One early study showed that the addition of albumin to a solution of mucin drastically increased the viscosity of the solution [67]. A later study demonstrated that MUC5B supramolecular organization was reversible and dependent on the solution calcium concentration [68].

Today it is known that mucins form complexes with numerous other biomolecules. These include components found naturally in the mucus such as Ca$^{2+}$ [68], lipids [69], trefoil factors [70], lysozyme [71], lactoferrin [71], amylase [71,72], proline-rich proteins [71,72], MG2 [71], gp-340 glycoprotein [73], statherins [72], histatins [72], IgG-binding protein [74,75], and sIgA [71].

Given that so many components associate naturally with mucin, it is not surprising that purified mucins do not replicate mucus [76]. In addition, a question has been raised that the purification conditions used in many studies actually destroy the native structures of the protein domains, thereby making them nonfunctional and unable to assist in normal mucus formation [68].

Mucin function: Implications for use in biomaterials field

The mucins constitute the main proteinaceous components of the mucus and are the main contributors to the cell glycocalyx. The normal functions ascribed to the mucus include lubrication for the passage of objects, maintenance of a hydrated layer over the epithelium, a barrier to pathogens and noxious substances and a permeable gel layer for the exchange of gases and nutrients with the underlying epithelium [54]. The cell glycocalyx have protective functions comparable to that of the mucus [77]. However, new insights in the field of medicine, including improved analytical techniques, have led to a deeper understanding of the mechanisms underlying mucin function in vivo. Thus, the simplistic view of mucins as passive, water-holding barrier components has changed to include highly specific in vivo processes where the mucins play central and active roles [78,79].

Among the observations that may strengthen the idea to use mucins as biomaterial coatings are: 1) mucins hold biomolecules with important protective and regulatory functions, especially growth factors, cytokines, lysozyme, lactoferrin, sIgA, and IgG-binding protein [71,75,79,80], 2) mucin do themselves contain domains (vWF-like and EGF-like) that resemble those found in other functional proteins [53,79], 3) mucin overexpression on cell surfaces produces highly protective layers which inhibits integrin-mediated cell adhe-
sion [81]. Of particular interest is the field of mucosal immunology; for an introduction see [82].

Mucins in fundamental and applied surface science

**Fundamental adsorption studies**

Although the high molar mass, and bulky and polydisperse nature of the mucins have complicated their controlled study, several studies have added to the fundamental understanding of how mucins interact with solid surfaces. Results for mucins of relevance to this thesis are described below. Unless stated, physiological buffer conditions (near-neutral pH, 150 mM salt) have been used.

*Bovine submaxillary gland mucin (BSM).* Owing to its early commercial availability, BSM was one of the first mucins to be extensively studied. Proust and co-workers [83] pioneered the field by studying the adsorption of radiolabelled BSM to differently oxidized polyethylene surfaces. They found that the adsorption was slow and that the amount of adsorbed BSM increased with the degree of polymer oxidation. Specifically, thick, partially desorbable, mucin layers were formed on the most oxidized surfaces. In two later studies [84,85] using the surface force apparatus technique (SFA), the same authors describe the surface interactions between BSM layers on mica in double-natured terms. That is, while the formed BSM surface layers initially were sterically repulsive and depended on the mucin surface density, long-range attractive forces were observed at low surface coverage. The latter observation was explained in terms of bridging of loosely bound mucin segments to bare surface areas on the opposite mica surface. Significant hysteresis occurred between first and subsequent contacts, and the compressed/washed film thickness was 12 nm for BSM adsorbed from 0.15 M salt at 0.1 mg/mL.

Another study performed with amine- and carboxylate functionalized polystyrene showed that the driving force for adsorption was nonionic and that BSM formed multilayers at low pH [86]. Lindh and co-workers studied BSM adsorption by means of ellipsometry on hydrophilic and hydrophobized silica [87]. These authors found that BSM generally had a stronger affinity for hydrophobic surfaces and that salt addition increased the BSM uptake on hydrophilic surfaces, interpreted in terms of reduced electrostatic repulsion.

Data from our own group [88] have shown BSM to adsorb on hydrophobic polystyrene in a flat conformation (4-5 nm) yielding a coverage of 2.3 mg/m². The formed mucin layer was shown to be stable with less than 2% desorption over 28 h.
A recent study performed with QCM-D showed that a preformed BSM layer compacts on the addition of salt (cations) of high concentration [89]. In case of cations of high valency (La3+) the compaction effect was irreversible.

Porcine gastric mucin (PGM). Durrer and co-workers showed that, in similarity with BSM, PGM adsorbed according to a nonionic mechanism and that low pH induced multilayer formation [86]. Studies performed by Lee et al [64] on the adsorption of PGM to PDMS showed that the mucin-surface interaction was hydrophobic in character. Specifically, low pH favored adsorption and was explained by the exposure of hydrophobic residues in the nonglycosylated part of the mucin. Malmsten and co-workers used SFA to show that PGM adsorbed in a flat conformation on hydrophobized mica (3-4 nm) and that the formed surface layer was stable and partially attractive [90]. The latter observation was explained by considerable relaxation on the surface, relating to this mucins high propensity to aggregate.

Human high molar mass salivary mucin, MG1 (MUC5B). Lindh and co-workers showed that MUC5B, with few exceptions, adsorbed as BSM to hydrophobized and hydrophilic silica [87]. Accordingly, the adsorbed amount was higher on the hydrophobic surface, indicating that the driving force for adsorption was hydrophobic interaction.

In conclusion of these previous studies, the mucins show good adhesivity with low desorption on hydrophobic surfaces. The driving force for adsorption is mainly hydrophobic interaction between the surface and hydrophobic residues in the nonglycosylated part of the mucin. The adhesion on hydrophilic surfaces is more difficult to predict but generally counteracted by electrostatic repulsion. Charge screening and reduction of pH promote adhesion. Generally, a loosely associated outer layer could be detected initially. This loose layer was desorbed upon rinsing, to give relaxed surface layers (i.e. compressed layers) with flat conformations. Layer thicknesses in the relaxed state ranged 3-34 nm; hydrophobic surfaces generally had thinner mucin layers than hydrophilic surfaces. Surface coverage was important for the layer properties; while mucin layers with high surface coverages acted sterically repulsive, mucin layers with low surface coverages could act adhesive.

Biomaterials
Although mucins have been in focus for long time in the pharmaceutical field, their role has mainly been to act models for the mucosal membranes. In the biomaterials field, there were relatively few studies involving mucin until the late 1990’s. In this sense, past work by Shi and co-workers have been somewhat pioneering. Specifically, these authors showed that coatings with BSM could prevent the adsorption of proteins, bacteria and cells to
polymeric surfaces of relevance to the biomaterials field [91,92]. In one particular facet of their work, they showed that mucin could be specifically extracted from a tear solution to facilitate for reduced bacterial uptake to a hydrophobic polymer [93]. Furthermore, of particular interest for the idea of using mucin for biomaterial coating purposes, a preliminary study showed BSM-coated polyurethane samples to be associated with low capsule formation and low inflammatory infiltration of host tissue after 30 days in a sheep [94].

In similarity with our group, Arnebrant and co-workers have developed the idea to combine mucins with components of biological interest. In their case, this involves the study of systems for use in dental applications. Studies by these authors [95] as well as by Dedinaite and co-workers [96] have shown that chitosan, a cationic polysaccharide, interacts readily with adsorbed mucin, and facilitates the build up mucin-chitosan polyelectrolyte multilayers (PEM). In continued studies by the former group, lactoperoxidase was incorporated in a mucin layer (MUC5B) using the same approach [97]. In a subsequent study they report that the specific enzymatic activity of lactoperoxidase is higher on gold surfaces precoated with human MUC5B than on bare gold surfaces [98].
Present investigation

Aim

Considering their biological functions and physicochemical properties, the mucins are interesting candidates for use as surface modifiers in biomaterial applications. The aim of the present work was three-fold: 1) to prepare a panel of defined mucin fractions for mucin coating biocompatibility studies, 2) to develop methods for the surface analysis of mucin-based coatings, and 3) to evaluate the effects of mucin precoating on the interactions of human neutrophils and proteins with a model biomaterial.

Materials and methods

Buffers and proteins

The following buffers were used throughout the work: 20 mM piperazine pH 5.0 supplemented with 0.15 mM NaCl (Pip20S), 50 mM sodium phosphate pH 7.0 supplemented with 150 mM NaCl (PB50S), 10 mM Tris-HCl pH 8.0 supplemented with 1 mM EDTA (TE), 50 mM Tris-HCl pH 7.0 supplemented with 150 mM NaCl and 0.05 vol % Tween 20 (TB50S-Tween20), and 20 mM sodium/potassium phosphate pH 7.4 with a total ionic strength of 150 mM (adjusted with NaCl) and a Na/K ratio of 33 (PBS20). High salt condition was generated using PBS20 with a NaCl-adjusted total ionic strength (PBS20-1M). For low pH conditions 20 mM sodium acetate of pH 4.0 with a total ionic strength of 150 mM (ABS20) was used. Alternatively, PBS20 was mixed in situ with 1 M HCl in a 10:1 ratio giving pH 1 (PBS20-pH1). The buffers were prepared by mixing the respective acid-base pairs in appropriate proportions. The ELLA washing and blocking steps involved use of PBS20 supplemented with 0.1 (ELLA-Wash) and 1 (ELLA-Block) wt % Pluronic F108 (BASF), respectively. For the corresponding ELISA steps, we used 50 mM Tris-HCl of pH 7.0 with 150 mM NaCl (TB50S) supplemented with 0.1 (ELISA-Wash) and 1 (ELISA-Block) wt % Pluronic F108, respectively. All cell experiments were performed in Hank’s balanced salt solution (HBSS; Sigma H8264).
The mucin starting materials were commercially available as lyophilized powders (BSM Sigma M3895 and PGM Sigma M1778) or collected directly from human saliva (MG1). The commercial BSM and PGM preparations had been prepared according to the methods of Tettamanti [99] and Glenister [100], respectively. Before fractionation, all mucin solutions were filtered and/or centrifuged to remove particulate matter.

All human single proteins were obtained from commercial sources and used without further purification. The used albumin preparations were as follow; essentially fatty acid-free, >99 % pure lyophilized human serum albumin (HSA; Sigma A3782), essentially globulin-free, ≥99 % pure lyophilized bovine serum albumin (BSA; Sigma A7638), and essentially fatty acid- and globulin-free, >99 % pure lyophilized porcine serum albumin (PSA; Sigma A1173). The used polyclonal IgG preparation (Sigma I4506) had a purity of >95 %, was prepared from pooled human serum, and supplied lyophilized and essentially salt-free. The used polyclonal sIgA preparation (Sigma I1010) had a purity of >95 %, was prepared from pooled normal colostrum, and supplied lyophilized and essentially salt-free. The used fibrinogen preparation (Sigma F4883) had a clottable fraction of ~95 %, was prepared from pooled human plasma, and supplied lyophilized and essentially plasmin-free. The human serum pool was prepared from 7 non-medicated, apparently healthy individuals, prepared as known in the art, stored frozen in aliquots at -80°C and thawed at 37°C immediately before use.

Below follows some background information on the non-mucin proteins. The focus is on their relevance as test substances in inflammation studies.

**Serum albumin** is produced in the liver and the predominant protein of blood [101,102]. Biologically, it has a colloidal-osmotic function and act as a carrier for molecules of low water solubility, including lipid-soluble hormones, bile salts, bilirubin, free fatty acids, calcium, iron and many drugs [101]. Serum albumin is a heart-shaped (equilateral triangle; 8x8x3 nm³) [103] protein with a $M_w$ of 66 kDa and an isoelectric point around 5.6 [104]. In contrast to many other serum proteins albumin is non-glycosylated. From a biomaterials point of view, albumin is generally considered a passivating agent, whose coatings lower the inflammatory response towards many materials [11,36,105]. However, long-term use of albumin-coated materials is complicated by the fact that significant exchange occur with other plasma proteins [36]. In addition, denaturation effects may limit the long-term use of albumin coatings [105].

**Immunoglobulin G (IgG)** is the second most common protein in blood, and synthesized and secreted by plasma B cells [102]. It has a $M_w$ of 150 kDa and is commonly described as a globular, Y-shaped (5x8x9 nm3) protein [106,107], with hypervariable domains functioning in antigen recognition.
IgG has strong implications to biomaterials research since it adsorbs strongly to many artificial materials, thereby labeling (opsonizing) them for detection by the host defense systems, primarily the complement system [17,18,108] and specific immune cells such as the neutrophils [109,110].

Fibrinogen (Fbg) [15,111] is produced in the liver and an important member of the coagulatory system, which normally respond to damaged blood vessels by forming blood clots. The coagulatory system is a cascade system, based on a series of events, involving the action of different proteins (coagulation factors) and platelets. Fibrinogen (coagulation factor I) is the precursor of fibrin, the nonsoluble protein component of the blood clot. Fibrinogen is a 340 kDa hexamer consisting of two sets of three different chains (α, β and γ) which link to each other with disulfide bonds [111]. Structurally, fibrinogen is an elongated molecule of 45 nm with two outer D-domains, each connected by a coiled segment to a central E-domain. [111] Fibrinogen has a central role in the inflammatory response to foreign materials. There are direct and indirect activation pathways. Firstly, platelets bind readily to surface-adsorbed fibrinogen/fibrin, thereby inducing neutrophil adhesion and activation [15]. Secondly, adsorption of fibrinogen to artificial surfaces could induce structural changes in the D-domains, leading to the exposure of the P1 epitope, which enables direct activation of neutrophils and macrophages via Mac-1 receptors [16,112]. The P1 epitope is also exposed in fibrin, adding a direct link between surface-induced coagulation and inflammation.

Secretory immunoglobulin A (sIgA) is a secretory protein existing in breast milk (colostrum) and mucosal secretions [113]. Dimeric sIgA is produced by plasma cells of the lamina propria and transported through the epithelial cells for secretion at the mucosal surfaces [113]. In contrast to serum IgA, secretory IgA carries the so-called secretory component (SC), which protects sIgA from proteolytic degradation in the enzymatically hostile mucosal environment [113]. The most common, dimeric form of sIgA has a $M_w$ of 380 kDa [106]. Secretory IgA plays a central at the mucosal surfaces where it captures and prevents pathogens from reaching the underlying epithelium. This protective mechanism is central in mucosal immunity and sIgA is therefore highly interesting when studying host-mucin interactions [113]. Neutrophils react strongly towards sIgA-opsonized materials [114] and sIgA has been suggested to trigger the complement system [115].

Serum is the cell-free, clotted fraction of blood. This complex mixture contains minerals, lipids, carbohydrates, and proteins. The total protein concentration of serum is ~70 mg/mL [116]. Since serum represents a complex mixture of most of the proteins governing inflammatory response in vivo, serum is a highly relevant model substance. In the present work 1 vol %
serum solutions were used. Although this may have influenced the relative serum composition, it should be noted that a previous study has shown that the amounts of HSA and IgG (the most abundant serum proteins) adsorbed from a 1 vol % solution onto a hydrophobic surface essentially resembled those adsorbed onto the same substrate at higher concentrations [117].

Surfaces and surface cleaning

Several different surfaces were used in this work; some acted as models and some had more real-type character. The model surfaces included non-derivatized and methylated (hydrophobized) silica, and polystyrene (micro-titer plates). The more real-type surfaces included Theranox (cover slips) and a panel of 10 polymers of relevance to the biomaterials field, namely polymethyl methacrylate (PMMA), polyethyleneterephthalate (PET), polybisphenol A carbonate (PBAC), polyvinylchloride (PVC), polystyrene (PS), polyethylene (PE), polypropylene (PP), polydimethyl siloxane (PDMS), polytetrafluoroethylene (PTFE), and amine-functionalized PTFE (NH-PTFE).

The silicon-based surfaces were prepared as follows. Silicon wafers (P-doped, resistivity 10-20 ohm cm; Wafernet) were cut along the <100> directions into suitable pieces. Surfaces were cleaned for 10 min in “piranha” solution consisting of 2:1 parts of concentrated H$_2$SO$_4$ (Fluka 30473) and 30 % H$_2$O$_2$ (Fluka 95313). The piranha cleaning was repeated 3 times. After rinsing with copious amounts of ultrapure water (18.2 Mohm, low organic content) the surfaces were either used immediately for the next-following modification steps or stored in ultrapure water until used. The cleaning was considered to give stable surfaces for 3 h if stored as mentioned. Immediately before use the surfaces were dried with N$_2$. This cleaning procedure resulted in surfaces with a thin silicon dioxide layer showing an initial water contact angle of 5º or less. In the following, these surfaces will be referred to as “hydrophilic silica”. Hydrophobic surfaces were prepared as follows: freshly piranha-cleaned silicon surfaces were rinsed 3 times in 99.7 % ethanol (Solveco Chemicals AB) and dry xylene (Riedel de Hahn 33817), respectively. After the final xylene rinse step the surfaces were transferred to 1 % dichlorodimethylsilane (Fluka 80430) in xylene and left there for 5 min under swirling. Thereafter 3 additional rinsings with xylene were performed; while still in the second rinse solution the surfaces were sonicated for 10 min and then rinsed a last time with xylene. Finally, all surfaces were rinsed 3 times with ethanol and left in ethanol until used. Modified surfaces were considered to be stable for 48 h if stored as mentioned. Immediately before use the surfaces were dried with N$_2$. The resulting methylated surfaces had initial water contact angles around 80º and are subsequently referred to as “hydrophobic silica”. The chemical identities of the two silicon-based model surfaces were confirmed using X-ray photoelectron spectroscopy (XPS).
addition to being positively identified, the surfaces showed low levels of organic contamination. The 10 polymers used in Paper I were of non-medical grade and supplied by a local supply house. After rubbing and washing in isopropanol (known to give low amounts of residual matter on evaporation) substrates were sonicated for 10 min in the same solvent. The sonicated substrates were then thoroughly rinsed with isopropanol and dried with N₂. Chemical identities were confirmed by means of XPS; of the 10 polymers, only PVC and PBAC contained a thin organic contamination layer. The polymers were all hydrophobic, with water contact angles in the range 64-110°. Thermanox (Nunc) is generally described as “oxidized polyethylene terephthalate (PET)” and should therefore be considered a relevant biomaterial model [4]. This material was used “as received” without further cleaning. The initial water contact angle for the used side of this material was approximately 75° when analyzed as received. However, one hour of equilibration (i.e. the time to reach at least near-plateau conditions during protein adsorption) in water rendered the surface more hydrophilic, with an initial contact angle of approximately 55°.

No analysis of surface roughness was performed. However, the methylated silica and Thermanox have been well characterized in previous studies and have surface roughnesses (500 x 500 nm²) of comparable orders (0.3-1.3 nm) [118,119].

Surface wettability

The surface wettability of each material was determined by means of static water contact angle measurement. This technique is based on the fact that a drop of water adopts the energetically most favorable shape when applied to a surface. From measuring the contact angle, i.e. the angle between an imaginary tangent drawn on the drop’s surface at the contact point and a tangent to the supporting surface, it is possible to determine the surface energy of the material [120]. Normally, wettability is expressed simply as the contact angle (in degrees), without calculation of the surface energy. Wettable (highly hydrophilic) surfaces have water contact angles typically <20° and non-wettable (highly hydrophobic) surfaces have water contact angles typically >80°.

The used equipment was a FTÅ200 goniometer (FirstTenÅngstroms). Measurements were performed with 10 μl of MilliQ-grade water and data were collected after 5 min of equilibration in a humid atmosphere. Alternatively, data were collected continuously over 5 min under ambient conditions and extrapolated to zero time for determination of the initial contact angle. Data were evaluated by the drop shape method [121] using a non-spherical fit algorithm. Reported values are means of 3 measurements, which include six individual contact angles.
Mucin fractionation and basic characterization

**Chromatographic fractionation**

Following pretreatment (centrifugation and/or filtration), the mucins were fractionated by chromatographic means. This mainly involved fractionation according to size (size-exclusion chromatography; SEC), but in case of the bovine mucin also involved prefractionation according to charge (anion-exchange chromatography; AEC). For introductions to these fractionation techniques, see [122] and [123]. All chromatographic steps were optimized in respect to sample load, buffer conditions, and flow rate. To minimize effects of hydrophobic interactions all operations were performed at 6°C. In general, glassware was used instead of plastics to minimize nonspecific adsorption. All chromatographic media were purchased from GE Healthcare.

**Biochemical characterization**

All mucin preparations were characterized using standard biochemical methods. These included amino acid analysis, thiol determination, carbohydrate analysis (pentose/hexose, sialic acid and GlcNAc/GalNAc), SDS-PAGE analysis, protease- and glycosidase assays, and albumin dot blot analysis. Below follows some comments on each of these methods.

**Amino acid analysis.** Corrections were made for the destruction of Thr, Ser and GlcNAc/GalNAc using standard recovery values of 0.96, 0.90 and 0.45, respectively. In the calculation of the weight recoveries of the amino sugars the residue molar mass for the acetylated form (203 Da) was used instead of that (161 Da) for the non-acetylated sugar. The BSM and MG1 amino acid data were corrected for albumin as determined by the albumin dot blot analysis (see below), using known BSA and HSA compositions [104].

**Thiol determination.** Free thiols were titrated using 2,2’-dipyridylidisulfide (2-PDS) essentially following the method of Stuchbury et al [124] Total thiol contents were determined from the amount of half-cystine equivalents as determined by amino acid analysis. Reference total thiol contents were calculated from literature primary sequence data [104] assuming linearly scale-able reference compositions, an average amino acid molar mass of 119 g/mol, and similar carbohydrate contents as reported here.

**Carbohydrate analysis.** The total amounts of pentose/hexose and sialic acid were analyzed essentially as described by Roe [125] and Svennerholm [126], using standards of D-galactose and N-acetylneuraminic acid, respectively. N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) were determined by amino acid analysis.
**SDS-PAGE analysis.** Sample compositions in the low molar mass region (30-1000 kDa) were analyzed by SDS-PAGE (PhastSystem; GE Healthcare) under reducing and non-reducing conditions according to standard protocols. Gel staining was performed using a complementary protein-carbohydrate staining protocol consisting of a slightly modified, combined periodic acid-Schiff base-silver staining [127]. Relative molar masses were deduced from comparison with commercial protein standards and multimer bands of albumin; although the limit of separation accordingly was 402 kDa, the separation media was considered to give rough mass estimates up to approximately 1000 kDa. An introduction to gel electrophoresis could be found in [128].

**Protease- and glycosidase assays.** The protease (Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride) - and glycosidase activities (Glc, Gal, Man, Fuc, GlcNAc and GalNAc) were determined in each mucin preparation. After incubation of the samples with the enzyme substrates overnight at room temperature, the enzymatic activities were determined from comparing the signal intensities of the sample/substrate mixture at 405 nm with the signal intensities of standard/substrate mixtures of known concentration.

**Albumin dot blot analysis.** The albumin content of respective mucin preparation was estimated using dot blot analysis. Primary targeting was performed using polyclonal antibodies directed against bovine, porcine and human serum albumin, respectively. Detection was performed using an alkaline phosphatase-conjugated secondary antibody - BCIP-NBT alkaline phosphatase substrate system. Serum albumins of bovine, porcine and human origin served as standards. Spot intensities, in turn translated into albumin contents, were quantified using a standard flatbed scanner.

**Biophysical characterization: Dynamic and static light scattering**

The light scattering techniques are based on the fact that particles (e.g. proteins) in a liquid medium scatter light following irradiation. In the present work both dynamic and static light scattering have been used. Static light scattering (SLS) measures the time-averaged total intensity of scattered light to give information about the weight-averaged molar mass ($M_w$), the second virial coefficient ($A_2$), and the root-mean-square radius ($R_{rms}$). In this work we combined SLS (in a multangle setup) with size-exclusion chromatography (SEC) and refractometry (RI). Dynamic light scattering (DLS) measures the variation in intensity of scattered light with time, which could be used to determine the diffusion constant ($D$), and thus the hydrodynamic radius $R_h$ of the particle. For more theoretical background on the SEC-MALS-RI and DLS techniques, please consult [129] and [130], respectively. Below follows short method descriptions.
SEC-MALS-RI. This technique was used to determine the weight-average molar mass ($M_w$), z-average root-mean-square radius ($R_{rms}$), polydispersity index ($M_w/M_n$), and fractional composition of each mucin preparation (Fig. 6).

Figure 6. Chromatographic profiles of a typical mucin fraction (BSM-II) presented as the combined light scattering (MALS) and refractive index (RI) signals as a function of total eluted volume, after gel filtration on an analytical Sephacryl S-1000 column. The vertical line distinguishes the monomer region from the void region, which contains multimers/aggregates. Superimposed are the calculated molar masses ($M_w$) and z-average root-mean-square radii ($R_{rms}$) for each elution volume. To allow for a good presentation, the RI and MALS signals have been multiplied by a factor 333.

The used MALS-RI equipment was supplied by Wyatt Technologies and consisted of a DAWN-EOS multiangle light scattering detector and an Optilab DSP differential refractometer. Samples were separated at a linear velocity of 19 cm/h using an analytical Sephacryl S-1000 SF column (1 x 30 cm$^2$) and the final analysis was performed using the Astra software (version 4.90; Wyatt Technologies). Following the methodology described by Andersson et al [131], detector data were fitted using first order polynomials. For the determination of $M_w$ and $R_{rms}$, Debye plots were constructed and extrapolated to zero detection angle according to the method of Berry [132]. Multimer analysis was performed using low-angle data [131]. The refractive index increments were determined for the bovine and porcine mucins using the Optilab DSP refractometer, yielding $dn/dc$ values of 0.132 and 0.163 mL/g, respectively. For the human mucin a literature $dn/dc$ value of 0.165 mL/g [133] was used.
Dynamic light scattering (DLS). All measurements were conducted using the setup and methodology described by Schillén et al [134] and Bastardo et al [135], respectively. Measurements were performed at 22 ± 0.2°C and data collected from at least 5 scattering angles in the interval 30-130º were evaluated using the GENDIST software. Values for the medium refractive index (n) and viscosity (η) were 1.33 and 955 μPas, respectively. Taking into account that some errors might have been introduced by uncertainties in these values, the calculated hydrodynamic radius was considered to be an “apparent” hydrodynamic radius.

Conformational analysis. Information on mucin conformation was obtained by calculating the $R_{rms}/R_h$ ratio ($\rho$) [135,136]. Typically, random coils generate $\rho$ values of 2.0 or higher, and more compact structures show lower values. Alternatively, the shape-dependent Mark-Houwink parameter $\alpha$ [61] was determined by constructing plots of log $R_{rms}$ against log $M_w$ and determining the slope of the best linear curve fits. Generally, while random coils have $\alpha$ values around 0.5, compact spheres would yield $\alpha$ values equal to 0.33 [61].

Surface analysis of mucin-based coatings

Bicinchoninic acid (BCA) protein assay

This method is based on the alkaline reduction of Cu$^{2+}$ to Cu$^+$ in the presence of peptide bonds and either of the four amino acids cysteine, cystine, tryptophan and tyrosine, and the subsequent complexation of Cu$^+$ with bicinchoninic acid (BCA), giving a complex with an absorbance maximum at 562 nm [137]. The working procedure included incubation of sample/standard with Micro BCA reagent (Pierce 23232), followed by 4 h incubation at 37°C and 48 h incubation at 8°C in sealed containers. The total protein uptakes on samples with known surface areas were determined from relating the absorbance of the liquid phase at 570 nm to standard curves constructed using solutions of known analyte concentrations.

Enzyme-linked immunosorbent (ELISA) and lectin (ELLA) assays

ELISA and ELLA are solid-phase techniques which rely on the sequential targeting and detection of surface-bound analytes. In case of ELISA, the primary targeting element is an antibody and in case of ELLA it is a lectin. Secondary targeting (detection) of the primary targeting element (antibody/lectin) is achieved by subsequent incubation with a substance with specific affinity for the primary targeting element. The second targeting element is linked to an enzyme, enabling for the detection of the primary target via coloring reactions. In the present work ELISA was used to detect HSA, IgG, sIgA, fibrinogen, and whole serum. The ELLA was based on the jacalin
lectin, which specifically recognizes the Thomsen-Friedenreich (Tn) epitope in mucins and other O-glycosylated proteins [138]. Below follows descriptions of the used ELLA and ELISA protocols.

**ELLA.** Coated substrates were blocked with ELLA-Block for 2 h and thereafter-washed 3 times using PBS20. Washed substrates were then incubated for 1 hour with biotinylated jacalin (Vector Labs B1155) at 1 μg/mL in ELLA-Wash, before being washed as above and incubated 1 hour with streptavidin-HRP conjugate (Vector Labs SA5004) at 0.5 μg/mL in ELLA-Wash. After 3 final washings, the samples were incubated for 5 min in clean cell culture plates with TMB peroxidase substrate (Sigma A3219). The color reaction was stopped by adding one equal volume of 1 % SDS to the sample solution. The amount of surface-bound mucin, represented by the amount of released substrate degradation product, was monitored at 405 nm using a benchtop spectrophotometer (Multiskan MS).

**ELISA.** The ELISA protocols were similar to the ELLA protocol, with the following exceptions. The used buffer system was TB50S and not PBS20. Furthermore, the primary incubation step involved the use of a polyclonal rabbit antibody (IgG) directed towards each human protein, i.e. anti-HSA (Sigma A3293), anti-kappa light chain IgG (Pharmacia 17-0502-01), anti-fibrinogen (DakoCytomation A0080) and anti-α chain IgA (Pharmacia 17-0499-01), respectively. For the whole serum experiments, a polyclonal rabbit anti-human whole serum IgG (Sigma H8765) was used. The secondary incubation step involved use of a monoclonal mouse anti-rabbit IgG-ALP conjugate (Sigma A2556). Antibodies and conjugate were diluted 1:2500 and 1:25000, respectively, in ELISA-Wash. For final development, a pNPP phosphatase substrate (Sigma S0942) was used. The color reaction was stopped after 25 min by the addition of one part 3 M NaOH to five parts sample solution. The amount of surface-associated protein was then quantified spectrophotometrically according to the above.

All ELLA and ELISA procedures were performed under ambient conditions.

**Ellipsometry**

Ellipsometry is a non-destructive, real-time optical technique that analyzes the dielectric properties (Fresnel equations; complex refractive index) of thin films. These qualities make ellipsometry most suitable to follow the adsorption of proteins to a surface. The underlying principle of ellipsometry is based on the fact that polarized light changes its state of polarization upon reflection at a sample surface. The two ellipsometric angles (“polarization angles”) Δ (relative phase change) and Ψ (relative amplitude change) are used to calculate the complex refractive index (N=n+ik) and the mean thick-
ness \( (d_{\text{eff}}) \) of the film. A review on ellipsometry as a tool in protein adsorption studies is found in [139].

In the present work, samples were analyzed in air, after rinsing with MilliQ-water and drying with N\textsubscript{2}, at an incident angle of 70° using an ELX-02C ellipsometer (DRE GmbH). The two polarization angles (\( \Delta \) and \( \Psi \)) were determined using an internal algorithm and the effective mean layer thickness \( (d_{\text{eff}}) \) and refractive index \( (n_{\text{eff}}) \) of the assumed uniform protein film were determined using a multi-layer model with the imaginary part of N set to zero. Refractive indices used for silicon dioxide and dichlorodimethylsilane were 1.457 and 1.465, respectively [140]. The mean layer thickness, based on at least 3 individual measurements, was translated into adsorbed mass using the formalism previously described by Cuypers et al [141]. In this study values of M/A, expressed in g/m\textsuperscript{3} and calculated as described elsewhere [141], were 4.38 and 4.14 for the mucins and the non-mucin proteins, respectively.

Alternatively, the mucin adsorption was studied in situ by measurements under stagnant conditions using a trapezoid quartz cuvette at an incident angle of 68°. After establishment of a baseline in PBS\textsubscript{20}, the bovine/porcine and human mucins were added to final concentrations of 0.25 and 0.05 mg/mL, respectively. After reaching a mass plateau, the surfaces were washed in situ with PBS\textsubscript{20} for 10 min at 20 mL/min. In similarity to the above, polarization data was processed using a multi-layer model. The PBS\textsubscript{20} buffer refractive index was determined to 1.334, using an Abbe refractometer. The mean layer thickness, based on at least two repetitions, was converted to adsorbed mass using the relationship described by de Feijter and co-workers [142]. After each run, the quartz cuvette was cleaned with 2 % Hellmanex II (Hellma GmbH) and 6 M HNO\textsubscript{3} for one hour and 10 min, respectively.

**Quartz crystal microbalance with dissipation monitoring (QCM-D)**

Upon applying an alternating voltage over a thin quartz crystal, the crystal starts to oscillate. The resonance frequency \( (f) \) of the crystal depends on the total oscillating mass, including water coupled to the oscillation. If a protein adsorbs to the crystal surface, the frequency decreases \( (\Delta f) \). If the adsorbed protein film is thin and rigid the decrease in frequency is proportional to the mass of the adsorbed film \( (\Delta m) \), and could be calculated using the Sauerbrey relation [143]:

\[
\Delta m = - \frac{C \times \Delta f}{n} \quad (2);
\]

where \( C \) is the mass sensitivity constant of the system and \( n \) is the frequency overtone number \((n = 1, 3, \ldots)\). The Sauerbrey relation holds so long as the adsorbed film is sufficiently thin and has low viscoelastic coupling with the
surrounding medium. However, for most proteins, the adsorbed layers do not simply adsorb as “dead” rigid mass but undergo deformation under shear oscillatory motion. As a consequence, the Sauerbrey relation becomes invalid. In such situations a Voight–Kelvin-based model may be used to describe the relationship taking into account viscosity and complex shear modulus of the layer. In the setup used in the present work, the dissipation of the crystal oscillations, $D$, was monitored. This parameter is defined as the ratio of the dissipated ($E_{\text{dissipated}}$) and stored ($E_{\text{stored}}$) energy according to the following:

$$D = \frac{E_{\text{dissipated}}}{2\pi E_{\text{stored}}}$$  \hspace{1cm} (3).

A rigid film displays little viscoelastic coupling and has a longer decay time, while a nonrigid film demonstrates significant viscoelastic coupling and a considerable decrease in decay time. Regardless of rigidity, adsorption of more mass decreases the frequency of oscillation.

In the present work, a Q-Sense D300 instrument (Q-Sense AB) was used to follow the real-time adsorption of proteins on polystyrene-coated QCM crystals (Q-Sense AB). Before each run, the sensor surfaces and tubings were cleaned with 0.1 M NaOH in 10 % ethanol for 30 min and thereafter thoroughly rinsed in MilliQ-grade water. The freshly cleaned surfaces were installed into the measuring chamber, which had been cleaned using 2 % Hellmanex (Hellma GmbH) and MilliQ-grade water, and equilibrated at 22 ± 0.01ºC with degassed PBS20 buffer until a stable baseline was reached. Thereafter, 0.5 mL of protein solution, equilibrated at 22°C, was injected into the chamber and allowed to adsorb until steady-state conditions were reached. Finally, 3-4 rinsings with PBS20 were performed in order to remove loosely bound material. The change in frequency ($\Delta f$) and energy dissipation ($\Delta D$) were monitored continuously during adsorption and plots of $\Delta D$ against $\Delta f$ were constructed to illustrate the viscoelastic properties of the adsorbed layer [107]. All presented $\Delta D$-$\Delta f$ plots are based on the normalized fifth overtone frequency ($f_5/5=25/5$ MHz).

The QTools software (Q-Sense AB) was used to model the effective hydrodynamic thickness ($d_f$), density ($\rho_f$), shear viscosity ($\eta_f$) and shear elastic modulus ($\mu_f$) of each coating. Specifically, the Voight-Kelvin based viscoelastic model of Voinova et al [144] was employed and values for the bulk fluid density and viscosity were 1000 kg/m$^3$ and 1 mPas, respectively. The layer density was allowed to adopt values between those of the bulk fluid and a theoretical close-packed protein layer (1400 kg/m$^3$) [145]. The relaxation time ($\tau_f$) of each surface layer was calculated as the ratio between the shear viscosity and the shear elastic modulus [146]. Furthermore, the coating water content was estimated from comparing “wet” QCM-D based and “dry” mBCA based (estimated as the protein surface concentration on a Thermanox substrate coated under the same conditions) adlayer masses.
X-ray photoelectron spectroscopy (XPS)

This technique relies on the fact that a material, upon irradiation with X-rays, emits photoelectrons which are characteristic of the atoms of the material’s top layer. Because the energy of a particular X-ray wavelength is known, we can determine the electron binding energy of each of the emitted electrons:

$$E_{\text{binding}} = E_{\text{photon}} + E_{\text{kinetic}} - \Phi$$  \hspace{1cm} (4);

where $E_{\text{binding}}$ is the energy of the electron emitted from one electron configuration within the atom, $E_{\text{photon}}$ is the energy of the X-ray photons being used, $E_{\text{kinetic}}$ is the kinetic energy of the emitted electron as measured by the instrument and $\Phi$ is a work function specific to the spectrometer.

The used equipment in the present work was an ESCA 300 spectrometer (Scienta Instruments AB) [147]. XPS data was acquired orthogonally to the sample using monochromized Al Kα X-ray radiation with the energy of 1487 eV. The pressure in the analysis chamber was typically below $1 \times 10^{-9}$ mbar. Survey spectra were collected for identification purposes and high-resolution spectra of nitrogen (N1s), a marker of proteinaceous material, were collected, linearly background-corrected and integrated for quantitative purposes. To minimize errors due to long-term radiation, the nitrogen spectra were acquired before the survey spectra. The effective thickness of the protein film was determined using an analogue to the Beer-Lambert relationship, assuming uniform film thickness and 100% surface coverage [148]:

$$d = -\lambda \cos \theta \ln \left(1 - \frac{I_p}{I_{p,\infty}}\right)$$  \hspace{1cm} (5);

where $I_p$ is the observed intensity of the peak corresponding to N1s in the protein, $I_{p,\infty}$ is the intensity of the N1s peak from a pure, “infinitely” thick protein sample (i.e. a sample which gives no signal from the underlying substrate), $d$ is the thickness of the protein film, $\lambda$ is the inelastic mean free path for the N1s photoelectron and $\theta$ is the photoelectron emission angle in relation to the surface normal. The used $\theta$ value was 0°, and $\lambda$ was calculated as 33 Å using the NIST Electron Effective-Attenuation-Length Database [149]. The cross-section of the N1s photoelectrons, relative that of the ionization of the carbon 1s state, was 1.80 [150]. As the XPS analysis is performed in a vacuum environment, the determined thickness corresponds to that of a dried-down protein layer. Accordingly, for conversion from thickness to amount of surface-adsorbed protein ($\Gamma$), we used a protein density of 1.4 g/mL [145].
Infrared spectroscopy – Multivariate data analysis (IRS-MVDA)
Principal Component Analysis (PCA) is a multivariate technique for transforming complex data in such a way that the most important or relevant information is made more apparent. This is accomplished by constructing a new set of variables, principal components (PCs) that are linear combinations of the original variables in the data set. For a more detailed discussion on PCA please consult reference [151].

In the present work, PCA was used to evaluate IR reflectance spectroscopy data collected on differently coated methylated silica samples. Specifically, samples were analyzed using a Nicolet Continuum Infrared Microscope (Thermo). Spectra, averaged from 16 scans, were collected in the range of 650-4000 wavenumbers at a resolution of 2 wavenumbers. Correction was made for the air background. Data were evaluated using the SIMCA software (Version 8.0; Umetrics AB). After data mean-centering and scaling to unit variance (block normalization), principal component analysis (PCA) was performed. Score scatter plots were constructed to discriminate between different samples. Hotelling’s t-test (p<0.05) was used to detect outliers.

Neutrophil interaction studies

Neutrophil preparation
Granulocytes, of which the neutrophils constitute the predominant fraction, were isolated from heparinized peripheral blood on the day of experiment from nonmedicated, apparently healthy individuals essentially as described by Håkansson and Venge [152]. Typically, neutrophil counts were in the range 28-45 x 10⁹ cells/L and cell contamination less than 10 %. Before use, cell suspensions were carefully diluted in room-tempered HBSS to an appropriate concentration. Neutrophils were used within 2-3 h after preparation.

Neutrophil activation assays
Neutrophil activation was assayed in either of two ways; either by measuring the release of reactive oxygen species (ROS) or by measuring the release of human neutrophil lipocalin (HNL), a marker of secondary granule release. The ROS assay is a real-time assay and the HNL assay an endpoint assay. In a preliminary experiment, myeloperoxidase (MPO), a marker of primary granule release was assayed. Short descriptions of the two first assays follow below.

ROS assay. The total (extra- and intracellular) neutrophil production of ROS following contact with differently coated samples was quantified using luminol-amplified chemiluminescence [153]. Coated substrates were rinsed
twice with HBSS and transferred to 24-well optiplates intended for chemiluminescent measurements (Perkin-Elmer). Neutrophils, added immediately in 0.5 mL aliquots to the rinsed substrates (325,000 cells/sample), were followed by 100 μl of luminol-horseradish peroxidase reagent (50 μM luminol and 4 U/mL horseradish peroxidase; purchased from Sigma) [153]. The real-time production of ROS was monitored using a Victor² 1420 luminescence meter (Wallac). Plates were incubated at 37°C between all measurements. The delay time between cell addition and the first reading was approximately 4 min. After completed measurements, cell suspensions were removed and the neutrophil-exposed surfaces fixed for the microscopic analyses using 1.5 % glutaraldehyde (Merck).

**HNL assay.** Coated substrates were rinsed twice with HBSS and transferred to 24-well plates intended for luminometric measurements (Perkin-Elmer). Neutrophils, immediately added in 0.5 mL aliquots (450,000 cells/sample) to the different substrates, were thereafter incubated for 60 min at 37°C. The release of secondary neutrophil granules, monitored as the level of human neutrophil lipocalin (HNL) in the cell-free medium, was subsequently assayed using the following procedure: The cell suspensions were transferred to ice-chilled EDTA-containing Ellerman tubes, to give a final EDTA concentration of 3.3 mM. Cells were then removed from all samples by centrifugation at 600 g for 5 min. All supernatants were collected by careful pipetting, mixed 1:2 with 0.3 % cetyl trimethylammonium bromide (CTAB) and frozen at -70°C until assayed for HNL [28].

Due to non-specific interaction of neutrophils with the supporting plastic material, the “baseline” (system induced) activation was assumed to be 30 % of the measured HNL and ROS signals for the non-coated and highly activating control. Accordingly, the presented data has been corrected to a corresponding degree. See Paper IV for more detailed discussion.

**Neutrophil adhesion and morphology**

The neutrophil adherence and morphology were analyzed using a scanning electron microscope (LEO Gemini 1530 SEM). In detail, fixed samples were quickly rinsed with MilliQ-grade water, dehydrated stepwise using water solutions of ethanol (50-100 vol %) and finally supercritically dried in liquid CO₂. After subsequent gold sputtering, samples were imaged in the secondary electron detection mode using an acceleration voltage of 2 kV. Alternatively, the fixed and water-rinsed substrates were stained with 5 % Giemsa stain (Sigma GS-500) for 30 min, rinsed quickly with water and dried before being evaluated by means of light microscopy (Carl Zeiss). Relative mean cell counts (C.C.) and mean cell areas (C.A.) compared to the non-coated substrate were analyzed using the ImageJ software (NIH; version 1.36b). Cells were counted per one microscopic view, which correlated to
approximately 0.02 mm² for the SEM micrographs and 0.3 mm² for the light microscopy images.

Statistics
Generally, the data is presented as the arithmetic mean ± 1 SD and is based on 3 or more individual measurements \((n \geq 3)\). When appropriate, levels of difference in significance were tested with the two-tailed non-paired Student’s \(t\)-test. Significance levels of \(*P<0.05\) and NS=not significant, were used; data not marked by any of the two levels in the figures are the \(t\)-test controls.
Results and discussion

Paper I


Past mucin studies from our group have mainly involved the use of bovine submaxillary gland mucin (BSM). In this work, we expand our past research platform by preparing two more precisely fractionated BSM products as well as mucins from porcine gastric (PGM) and human salivary (MG1) sources. The rationale behind adding mucins from porcine and human sources was to address regulatory issues related to the medical use of bovine tissue materials. Furthermore, the use of different mucins makes it possible to study the effect of compositional and structural differences on the dissolved state, adsorption event and final mucin coating properties. Ultimately, such knowledge could be used to develop a greater understanding of the factors governing the performance of mucin-coated materials in vivo.

Fractionation and biophysicochemical characterization

Prefractionation by anion exchange chromatography revealed a substantial batch-to-batch variation in the composition of the Sigma M3895 BSM (Fig. 7). The observed variation was explained by significantly different albumin and aggregate contents in the studied BSM batches. Based on the mass analysis (mBCA) of the eluted fractions, the content of free albumin in batch 64H7170 was ~22 wt % (after centrifugation).
Prefractionation of commercial bovine submaxillary gland mucin (BSM; Sigma M3895). Chromatographic profiles are presented for equal loads of two different BSM batches (Batch 1: Lot 013K7028 and Batch 2: Lot 64H7170) separated on a Q Sepharose HP anion exchange chromatography column equilibrated in Pip20S buffer at pH 5.0. The A214 and conductivity signals are shown as functions of the total eluted volume. Adsorbed material was step-eluted using NaCl and BSM was collected for further fractionation at an elution volume of ~600 mL.

From an adsorption point of view, the presence of mucin aggregates and non-mucin components (e.g. albumin) introduces an uncontrollable factor. Specifically, these components are likely to influence the surface-binding event and ultimately have an impact on the formed adlayer. However, from a functional point of view, a distinction should be made between components free in solution and those in complex with mucin. In particular, the free and in most cases smaller non-mucin components are thought to have a competitive edge over the bulky mucin molecules in reaching and adsorbing to the surface, thereby preventing the mucin from efficient formation of a surface layer. However, the mucin-associated components are of potential importance for mucin function, in view of the fact that mucins capture and hold many important biomolecules \textit{in vivo}. It follows that while free non-mucin components are non-desirable, natural components associated with the mucin are considered to bring additional values to the mucin function.

To eliminate free non-mucin components, while inflicting minimal damage to the natural conformations and/or components in complex with the mucins, “mild” fractionation strategies were employed. This involved simple gel filtration protocols without use of reducing and dissociating agents. To enable for comparisons the BSM and PGM mucins were collected as high- and low molar mass variants, labelled by indices I and II, respectively.
The final preparations were positively identified as mucins by their polydisperse nature, large sizes ($M_w \geq 1$ MDa), characteristic amino acid compositions and high carbohydrate contents. Notably, the BSM contained a substantially higher fraction of sialic acid (36 wt %) than the other mucins (2-3 wt %). The main mucin components were suggested to be BSM1 gene translation product (BSM), pepsin-digested pig gastric mucin (PGM) and MUC5B gene translation product (MG1). While the BSM and PGM contained essentially no contaminant material, the MG1 appeared complex with a substantial content of non-mucin components. Our combined results indicated these components to bind to the mucin portion of the MG1 preparation (MUC5B) and not to exist free in solution. Specific protein analysis revealed 1.6 wt % albumin in the MG1 preparation. Under the used conditions, essentially no glycosidase (6 substrate specificities) or protease (general substrate) activities could be detected in the mucin preparations.

Light scattering experiments showed that the mucins were stable over 24 h in solution at room temperature, and that the average molar masses and root-mean-square radii for the predominant BSM, PGM and MG1 species spanned 0.8-4.2 MDa and 46-86 nm, respectively. In contrast to the other, random-coiled mucins, the high molar mass PGM and the MG1 appeared compact in solution (Fig. 8). In case of MG1, this was suggested to link to natural components associated with the mucin molecule.

![Figure 8](image.png)

*Figure 8. Mucin conformation analysis: log $R_{rms}$ plotted against log $M_w$ for the predominant mucin species. The calculated shape-dependent slopes $\alpha$ are found within parentheses. Generally, while random coils have $\alpha$ values around 0.5, compact spheres would yield $\alpha$ values equal to 0.33. ($n=2-3$)*
Mucin adsorption on hydrophilic and hydrophobic model surfaces

Ellipsometry was used to follow the adsorption of each mucin fraction on hydrophilic and hydrophobic silicon-based model surfaces (Fig. 9). All experiments were conducted using physiological buffer conditions.

Figure 9. Mucin adsorption on hydrophilic (a) and hydrophobic (b) silica. Mass uptake is presented as a function of mucin concentration after ~24 h adsorption.

On the hydrophilic surface [Fig. 9(a)] mean mass uptakes for the BSM and PGM fractions ranged 0.3-1.1 mg/m² after ~24 h of adsorption at 5 mg/mL. The high molar mass variant of respective mucin showed the highest mass uptake. In addition, these mucin variants showed no distinct mass plateaus in the used concentration range, contrary to their low molar mass counterparts. The latter observations indicated that size is a factor influencing mass uptake. The MG1 fraction, which was only adsorbed at 0.25 mg/mL due to limited availability, produced a surface coverage of 1.9 mg/m². The high mass uptake of the MG1 was suggested to relate to its large size, compact
conformation and complex composition. All mucins adsorbed slowly to the surfaces; 10 h or more were needed to reach steady-state conditions.

On the hydrophobic surface [Fig. 9(b)] mass uptakes were considerably higher than on the hydrophilic surface, a difference that was suggested to stem from less pronounced hydrophobic interaction/more pronounced charge repulsion on the hydrophilic surface. Similar findings regarding mucin uptake on hydrophilic and hydrophobic surfaces have been made previously [87]. Mean mass uptakes on the hydrophobic surface after 24 h of adsorption reached 2.6, 2.6 and 5.0 mg/m² for the BSM, PGM and MG1 mucins, respectively. Notably, the BSM fractions contrasted the other mucins by showing more pronounced concentration dependence. This finding was suggested to relate to the relatively high sialic acid content of the BSM mucin, which should lead to stronger mucin-mucin repulsion at high surface coverages. Kinetic analysis of the initial adsorption on the hydrophobic surface showed that high molar mass mucin species upon compaction could play important roles in populating a surface. These findings stressed the importance of characterizing mucins used in comparative adsorption studies, not only with respect to protein contamination but also with respect to multimer/aggregate content.

In situ measurements performed on the hydrophobic surfaces showed that, for the BSM mucins the effective ellipsometric thicknesses correlated with the root-mean-square radii \(R_{rms}\) of the predominant adsorbing mucin species. For the low molar mass PGM variant, disproportionately thick surface layers were observed. This was believed to relate to the porcine mucin’s strong propensity to self-interact and form multilayers [90]. In case of MG1 and the high molar mass PGM, the ellipsometric thicknesses were lower than the \(R_{rms}\) predicted. For the MG1 mucin, this was suggested to relate to non-mucin proteins associated with the mucin molecule.

**Mucin adsorption on polymers of relevance to the biomaterials field**

In an attempt to link adlayer properties on the silicon-based model surfaces to those expected for polymeric materials used within the biomaterials field, a screening experiment was performed with a panel of polymers of different wettability.

Polymer substrates were exposed overnight at 37ºC to a BSM fraction dissolved at 0.5 mg/mL, and thereafter washed, either by mild rinsing or by thorough wash at high shear rate (50 s⁻¹). The substrates were then analyzed with respect to wettability. It was found that (Fig. 10), all polymers except PDMS showed significantly lower water contact angles after mucin adsorption and use of the mild washing procedure (14-51 % increased wettability).
When high shear was applied, contact angles increased for the majority of polymers. Still, these contact angles were substantially lower than those for the bare controls. Although contact angle measurements cannot determine the mass uptakes on the surfaces, these results indicated that relatively strong interactions were developed between the mucin and most of the polymers. In turn, this was promising for future mucin biocompatibility studies performed with polymeric materials of interest to the biomaterials field.

Figure 10. Static water contact angles for a panel of 10 polymeric materials, before and after adsorption of a BSM fraction. After adsorption overnight at 0.5 mg/mL and 37°C, washing was performed using low and high shear conditions. Values are means of 3 measurements and error bars represent mean ± 1 SD.

Paper II


Of fundamental importance to the understanding of the factors underlying the bioperformance of mucin-based coatings, is detailed knowledge about coating concentration and composition. Many surface-sensitive techniques are strongly limited by the demand for special experimental conditions or by low availability/ usability due to rare designs and/or complicated data analysis. The aim of the present work was to develop simple protocols based on available techniques for the quantitative and qualitative analysis of pure and complex mucin coatings.
For the determination of total protein uptake, we choose to use the Micro BCA (mBCA) protein quantification assay [137]. Figure 11 demonstrates the performance of the mBCA assay, by revealing a positive linear correlation between the mucin surface concentration on Thermanox and the molar mass of the adsorbing mucin species. Considering the different origins of the used mucins, the observed relationship suggested that mucin adsorption is governed by similar adsorption mechanisms. This finding is well supported by the general view of mucins as surface-active biomolecules sharing largely similar physico-chemical properties [54].

Figure 11. Mucin surface concentration (Γ) on Thermanox substrates as a function of molar mass (Mw) of the adsorbing mucin species. Substrates were spot-coated with the BSM-I, BSM-II, PGM-I, PGM-II and MG1 mucin fractions at similar coating concentrations (0.13-0.25 mg/mL in PBS20). All substrates were analyzed using the mBCA assay. (n=3)

To improve sensitivity and enable compositional analysis of complex mucin coatings, we complemented the mBCA assay with mass-calibrated, enzyme-amplified assays specific for mucins and human host proteins. We found that while the mBCA assay was of limited use at low surface concentrations, steric effects influenced the lectin- and antibody based assays at high surface layer densities. Promisingly, non-specific responses due to substrate interaction were low for the latter assays. Cross-reactions were observed during ELLA analysis of surface analytes sharing high degree of O-glycosylation.

Our results showed that mucin desorption upon addition of common human host proteins is low on the used polymeric material (Thermanox). The combined results suggested that apparent low levels of non-mucin proteins in the complex mucin coatings mainly could be interpreted in terms of reduced exposure of proteins to the bulk environment (Fig. 12). Based on this, a methodology was presented for determination of the fraction of surface-
exposed, presumed bioactive proteins in a complex mucin coating. Specifically, the fraction of surface-exposed protein was calculated as the ratio between the amounts of protein determined by the mass-calibrated ELISA assay (exposed protein) and the combined mBCA-ELLA-ELISA assay (total protein), respectively.

**Figure 12.** Analysis of complex mucin coatings on Thermanox. HSA content in mucin-HSA composite coatings analyzed by means of combined mBCA-ELLA-ELISA assay and mass-calibrated ELISA assay, respectively (a). Error bars represent mean ± 1 SD and significant differences compared to the mBCA-ELLA-ELISA-determined mass uptakes are marked for the ELISA data, using the following significance levels: *$P<0.05$ and NS = not significant. ELISA-determined protein contents in the mucin-protein composites compared to the single-coated protein controls as a function of molar mass ($M_w$) of the adsorbing protein species (b). (n=3)
Finally, X-ray photoelectron spectroscopy and infrared reflectance spectroscopy combined with multivariate data analysis were proven useful in the evaluation of mucin-based surface films.

Papers III and IV


Neutrophils are important mediators of biomaterial-induced inflammation [12,29,110,112]. In these works, we studied the interaction of neutrophils with a polyethylene terephthalate-based model biomaterial (Thermanox) coated under different conditions with BSM, PGM and MG1. Correlations were made between the neutrophil activation and the individual mucin uptakes on the substrates. In complement to other studies, we employed the QCM-D technique to evaluate possible differences in organization between different mucin coatings. Finally, to link to the behavior of mucin-coated materials in vivo, the neutrophil response towards mucin-coated substrates incubated with human serum was studied.

Neutrophil morphology and activation on mucin-coated surfaces

Figure 13(a) shows images of differently mucin-coated Thermanox substrates after 55 min of incubation with neutrophils. Clearly, coating with BSM, PGM and MG1 could be used to effectively suppress the adhesion of neutrophils to the surface. This finding was well supported by the ROS analysis. Neutrophil morphologies found on surfaces coated with mucins under optimal conditions resembled those observed for non-activated neutrophils found in circulation. Notably, low neutrophil adhesion could be obtained at a significantly lower coating concentration (0.125 mg/mL) for the compositionally complex MG1 mucin than for the relatively pure BSM and PGM mucins (1 mg/mL). A control experiment [Fig. 13(b)] demonstrated that the surface-passivating effect of MG1 not could be explained by its albumin contaminant.
Figure 13. SEM- and light microscopy images of Thermanox substrates coated with bovine (BSM), porcine (PGM) and human (MG1) mucins after 55 min of neutrophil exposure. Non-coated control (PBS20) is shown for comparison. While the MG1 mucin was adsorbed at one concentration (0.125 mg/mL), the BSM and PGM
mucins were adsorbed at one low (0.25 mg/mL) and one high (1 mg/mL) concentration. Neutrophils on Thermanox substrates coated with human serum albumin (HSA) at a concentration of 0.125 mg/mL (b). The relative numbers denote mean cell counts (C.C.) and mean cell areas (C.A.) ± 1 standard deviation compared to the non-coated material (PBS20 buffer control). All SEM imaging was performed in the secondary electron detection mode using an acceleration voltage of 2 kV. Insets show Giemsa-stained neutrophils imaged by light microscopy. Scale bars represent 10 μm (white) and 100 μm (black), respectively. See Materials and methods section for further details.

An alternative and methodologically interesting route to produce surface-passivating surfaces was to adsorb the mucins at low pH. In that way, lower mucin concentrations could be used. The success of this route was believed to relate to more compact packing of mucin on the surface due to aggregation/gelation effects.

**Analysis of mucin coatings**

Except for the high molar mass PGM variant, which was believed to be influenced by aggregation effects, the relative mucin uptakes on Thermanox (Fig. 14) correlated well with the relative uptakes described in Paper I for a hydrophobic model surface. Accordingly, we note the high mass uptake of the MG1 mucin and the concentration-dependent mass uptakes of the BSM and PGM mucins. In addition, it was shown that BSM and PGM adsorption under non-regular conditions, i.e. at higher mucin concentration, at higher ionic strength and at pH 1, led to higher mucin uptakes.
Figure 14. Mucin surface concentrations on differently coated Thermanox substrates. Regular coatings of BSM, PGM, and MG1, respectively, formulated at regular mucin concentration (0.125 or 0.25 mg/mL) (a). Non-regular coatings of BSM and PGM (low molar mass variants; index II omitted for improved readability), respectively, formulated at high mucin concentration (1.0 mg/mL), low pH (pH 1 and pH 4) and high ionic strength (1 M salt), respectively (b). Samples were analyzed using the mBCA assay. Significant differences compared to the regular mucin coatings are marked with asterisks (b), using the following significance levels: *$P<0.05$ and NS=not significant. Error bars represent mean ± 1 SD. ($n=3$)

Interestingly, no clear trend was found between the amount of adsorbed mucin and the degree of induced neutrophil activation. Specifically, substrates coated with mucins at high ionic strength showed significantly higher neutrophil activation levels than their corresponding substrates coated at high mucin concentration and at pH 1, despite the fact that all these substrates were coated with comparable amounts of mucin. From this, we con-
cluded that the amount of adsorbed mucin is no decisive marker of mucin coating performance.

Although performed with a different model substrate, the QCM-D analysis (Fig. 15) provided valuable structural information about the mucin coatings. Firstly, we found that the modeled thicknesses of the BSM (46 nm) and MG1 (93 nm) surface layers were in good agreement with the $R_{\text{rms}}$ values of the adsorbing mucin species (46 and 86 nm, respectively) described in Paper I. In contrast, the PGM mucin formed a considerably thicker surface layer (77 nm) than its $R_{\text{rms}}$ value (47 nm) predicted. This difference was suggested to be due to PGM multilayer formation, caused by more pronounced PGM self-association.

![Number 15](image)

*Figure 15. QCM-D evaluation of mucin coating properties. Diagram showing the final shifts in dissipated energy ($\Delta D$) recorded for given final shifts in frequency ($\Delta f$) for different mucin coatings on a polystyrene model surface. All mucin coatings were formulated according to Table 1 and studied in their final relaxed states after 3 rinsings with PBS20. Line constructs indicate the $\Delta D/\Delta f$ slope associated with each coating. Human serum albumin (HSA) adsorbed at 0.125 mg/mL for 20 h served as a reference.*

The QCM-D analysis helped link mucin coating bioperformance to mucin surface conformation. Specifically, the QCM-D data suggested that effective surface passivation could be obtained by either forming thick expanded mucin coatings (“BSM pH 1”) or by forming thin compact mucin coatings (“BSM Hi conc.”). Although the biological significance is unclear, it is tempting to discuss these structurally different layer conformations in terms of the two main layers of the native mucosa. That is, while the outer secreted layer of the mucosa is thick with a complex, expanded conformation [56],

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the inner cell-bound layer adopts a more ordered, relatively thin conformation [57]. A similar two-layer structure has been demonstrated in a study on adsorbed human saliva [154]. A recent study on the structure of mucus in colon suggests a link to mucin function; while the outer expanded mucus layer was colonized with many bacteria, the inner dense layer was essentially free from bacteria [155].

Since the electrostatic repulsion between the adsorbed mucin and the neutrophils should be largely screened at the high ionic strength used (150 mM) in Paper IV, surface-shielding through steric hindrance was suggested to be the most critical factor determining mucin coating performance. In the given context, the relatively thick and dense conformation of the MG1 coating, well explained its excellent performance. Possibly, the compact solution conformation and complex composition of the MG1 mucin favored the formation of biocompatible coatings. Particularly, we could not exclude the possibility that non-mucin components, associated with the MG1 mucin, possessed immune-suppressing capabilities.

**Effect of serum addition on the neutrophil response towards mucin-coated surfaces**

Very few neutrophils, all with non-activated morphology (round without signs of extracellular processes), were found on substrates bearing combined mucin-serum coatings (Fig. 16). The ROS analysis supported these observations.
Figure 16. SEM images showing Thermanox substrates coated sequentially with mucin (BSM or PGM) and human serum (1 vol %) after 55 min of neutrophil exposure. Serum-coated control is shown for comparison. Detailed coating procedures are found in Table 1 of Paper IV. The relative numbers refer to mean cell counts (C.C.) and mean spreading (contact areas; C.A.) compared to the non-coated control (PBS20). Scale bar represent 20 μm. (3n-4n)

Interestingly, the mucin-serum composite coatings were associated with lower ROS production, cell adhesion and cell spreading than the coatings formed from their constituent components. Plausible explanations to the improved performance of the mucin coating upon serum addition were: (a) the serum components adsorbed to non-coated regions on the mucin-coated surface, thereby reducing total neutrophil-substrate contact and/or (b) the serum components adsorbed specifically to the mucin thereby forming surface arrangements with reduced activation potential and/or (c) the serum
components adsorbed selectively to the mucin thereby leaving neutrophil-activating components behind in the bulk medium. Considering the diffuse organization of the original mucin coatings (Fig. 15), alternative (a) seems likely to explain a large part of the observed effect. However, given the large reductions in ROS and the relatively small differences in serum content and total adsorbed mass between the composites and the highly activating serum control, additional factors were suggested to influence composite coating performance. In line with alternatives (b) and (c), we highlighted the possibility that pre-adsorbed mucins could provide favorable support for adsorbing host components.

Paper V


In this study the specific interaction of bovine serum albumin (BSA), extracted from the BSM starting material (as described in Paper I), with BSM adsorbed on polystyrene was studied by means of ellipsometry and QCM-D.

We showed that albumin and mucin have significantly different adsorption properties. That is, while BSA adsorbed in a flat rigid layer the mucin adsorbed in a diffuse, highly viscoelastic layer. Subsequent addition of BSA to a preadsorbed mucin layer resulted in stiffening of the layer which was attributed to complexation of the mucin by BSA. In contrast, a preadsorbed layer of BSA prevented mucin adsorption altogether.

Combined mixtures of mucin and BSA in well-defined ratios revealed intermediate viscoelastic properties between the two separate protein species which varied systematically with the ratios of the proteins (Fig. 17).
Figure 17. Change of dissipation ($\Delta D$) versus frequency ($\Delta f$) for the 5th overtone during the adsorption of proteins on polystyrene. The numbers alongside the curves refer to different additions of proteins according to the following: (1a) BSM (0.05 mg/mL) followed by (1b) BSA (0.05 mg/mL) followed by (1c) rinsing in buffer; (2a) BSM (0.05 mg/mL) followed by (2b) BSA (0.025 mg/mL) followed by (2c) rinsing in buffer; (3) 1:¼ mixture of BSM (0.05 mg/mL) and BSA (0.0125 mg/mL); (4) 1:1 mixture of BSM (0.05 mg/mL) and BSA (0.05 mg/mL); (5) 1:½ mixture of BSM (0.05 mg/mL) and BSA (0.025 mg/mL); (6) BSA (0.05 mg/mL) only.

The results of this work shed light on the synergistic effects of complexation of lower molecular weight biomolecular species with mucin. Of practical interest to biomaterial applications, is the possibility to selectively control protein uptake and tailor the physical properties of the adsorbed layer.

Paper VI


The mucins provide the organizing framework of the mucus gel where they act in concert with many other biomolecules. In this work, we studied the effect of mucin precoating on the conformation and neutrophil-activating properties of some important host proteins adsorbed to model materials.

Neutrophil morphology and activation

Microscopy combined with neutrophil activation assays for the neutrophil releases of ROS and HNL, showed that mucin precoating greatly could reduce the strong immune-response normally induced by adsorbed immu-
noglobulin G (IgG) and secretory immunoglobulin A (sIgA), respectively. A similar finding was made for the proinflammatory fibrinogen. See Figure 18.

![Figure 18. Diagrams showing the relative amounts of HNL for Thermanox substrates coated sequentially with mucin (BSM, PGM or MG1) and protein (HSA, IgG, sIgA or Fbg), compared to the non-coated substrate. All coatings were formulated according to Table I. Error bars represent mean ± 1 SD and significant differences compared to the protein-coated controls are marked, using the following significance levels: * $p<0.05$ and NS = not significant. (2n-3n).](image)

**Analysis of complex mucin coatings**

Although the total mass uptakes of all studied proteins depended on the mucin surface concentration, a detailed composite analysis suggested the fraction of surface-exposed protein to be a stronger determinant of coating performance (Fig. 19)
Figure 19. Diagram showing the surface concentrations of total and exposed IgG for different mucin-IgG composites (a). HNL levels as a function of amount of exposed proteins for the BSM- and PGM-based composites (b). Significant differences compared to the protein-coated control were marked in (a) using the following significance levels: *$p < 0.05$ and NS = not significant. Error bars represent mean ± 1 SD for the total surface concentration. ($n=3$)

The unexpectedly low neutrophil activation showed by composites containing near-monolayer concentrations of exposed IgG and sIgA, respectively, suggested these to act synergistically with mucin on the surface. In support of this hypothesis, QCM-D measurements on a polymeric model surface (Fig. 20) revealed that a preadsorbed BSM layer could stabilize IgG through complexation.
Altogether, our data suggested that the unexpectedly good performance of 
the mucin-IgG and mucin-sIgA composites at least in part results from a 
different protein conformation adopted in the mucin layer. Since, it is gener-
ally accepted that protein adsorption to a polymeric surface leads to confor-
mational changes in the protein structure, we tentatively suggested that IgG 
and sIgA as well as fibrinogen, retain more native conformations when ad-
sorbed to mucin precoated surfaces than when adsorbed to bare surfaces. These findings are well supported by a recent study on a lactoperoxidase-mucin-gold system [98], which showed that the specific enzymatic activity of lactoperoxidase is higher on a gold surface precoated with human MUC5B mucin than on a bare gold surface. An interesting observation made in that work was that the described effect only appeared for mucin layers of relatively low surface concentration. Combined with the results of Paper VI, which indicated that mucin layers of relatively low surface density could act stabilizing on proteins, it is strongly suggested that sufficient penetration of the mucin layer is important for the synergistic effect to occur.

We propose a mutual interaction between the surface, the mucin layer and the arriving protein, in which the mucin layer can act stabilizing and prevent restructuring on the surface. Figure 21 shows a schematic representation of possible differences in the IgG adsorption to a bare surface and to a surface bearing a preadsorbed mucin layer. Specifically, Figure 8a illustrates how the IgG molecules adsorb freely to the bare surface, which interacts strongly with the arriving proteins causing a significant change in conformation. The effect of the mucin layer is proposed to be three-fold (Fig. 8b): Firstly, the preadsorbed mucin layer reduces the IgG uptake by hindering the IgG from adsorbing freely to the surface. Secondly, the mucin component lowers the accessibility of the adsorbed IgG by steric interference. Finally, the mucin acts stabilizing on the IgG molecule, thereby minimizing IgG restructuring on the surface.
Figure 21. Schematic representation of possible differences in IgG adsorption to a bare (a) and a mucin precoated (b) surface. Gray ellipses mark non-glycosylated hydrophobic domains interspersed between heavily glycosylated domains in the mucin molecule. Coating properties according to Table III in Paper VI are indicated.

Conclusions
We described simple routes for the preparation of one complex human salivary mucin (MG1) and four relatively pure bovine salivary (BSM) and porcine gastric (PGM) mucins. Since significant batch-to-batch variations were observed for the commercial BSM starting material, the importance of standardizing mucins used for comparative purposes was stressed. The reported MG1 preparation contained a substantial amount of mucin-bound components, including 1.6 wt % albumin. Light scattering experiments confirmed the existence of large, high molar mass mucin species. Average molar masses and root-mean-square radii for the predominant BSM, PGM and MG1 species spanned 0.8-4.2 MDa and 46-86 nm, respectively. In general, the mucins adopted random coil conformations in solution; however, the high molar mass PGM and MG1 appeared compact. Mucin adsorption on hydrophilic and hydrophobic model surfaces was a function of charge, size, conformation and compositional complexity. Adsorption was relatively slow
on both model surfaces and mass uptakes on the hydrophobic model equal
2.6, 2.6 and 5.0 mg/m², for BSM, PGM and MG1, respectively. Through a
screening experiment, we indicated that stable mucin coatings could be
formed on polymers of different wettability.

We demonstrated how a common protein quantification assay (mBCA)
could be used in combination with mucin- and human protein-specific assays
to determine the composition of complex mucin-based coatings. Specifi-
cally, we presented a method for the determination of the fraction of surface-
exposed, presumed bioactive proteins in a complex mucin coating. Our re-
sults showed that mucin desorption upon addition of common human host
proteins is low on a polymeric model material (Thermanox). Furthermore,
X-ray photoelectron spectroscopy (XPS) and combined infrared reflectance
spectroscopy-multivariate data analysis (IRS-MVDA) were proven powerful
tools in the evaluation of mucin-based surface coatings.

We showed that precoating with bovine (BSM), porcine (PGM) and human
(MG1) mucins could be used to effectively suppress the neutrophil response
towards a polymeric model biomaterial. By altering the coating concen-
tration we could achieve a similar suppressing power, which seems independ-
ent of mucin source. This indicated a high degree of functional homology
between the mucins. Neutrophils found on the mucin-coated substrates, were
small and round, resembling non-activated neutrophils found in circulation.
QCM-D analysis suggested efficient mucin surface-shielding to be critical
for good mucin coating performance. Interestingly, the shielding power ap-
peared equal for thick expanded and thin compact mucin coatings. Finally,
combined mucin-serum coatings were associated with low neutrophil activa-
tion.

The adsorption profile and viscoelastic properties of BSM, albumin (BSA)
and mixtures of the two proteins were studied using QCM-D. The adsorbed
proteins layers showed significantly different viscoelastic properties with the
BSM adsorbing in a thick, hydrated, diffuse layer and the BSA adsorbing in
a thin rigid layer. Preadsorbed layers of BSA prevented subsequent mucin
adsorption altogether which was surprising given that the albumin was ex-
tracted directly from the same source as the mucin. In contrast, secondary
addition of BSA to adsorbed mucin layers resulted in complexation of the
albumin with mucin leading to dramatically increased rigidity of the layer.
Mixtures of BSM and BSA showed properties intermediate of the two pro-
teins adsorbed separately with a systematic variation in properties depending
on the ratio of the two proteins in the mixture.

Finally, it was shown that mucins could be used to effectively reduce the
neutrophil response towards adsorbed proinflammatory proteins such as IgG,
sIgA and fibrinogen. Detailed analysis showed that the fraction of surface-exposed protein has a strong influence on the activation level. Interestingly, the mucin-IgG and mucin-sIgA composites showed exceedingly low degrees of activation, although containing near-monolayer concentrations of exposed IgG and sIgA. Conformational analysis by QCM-D suggested that the mucin component interacts actively with the arriving proteins, influencing the properties of the adsorbed proteins. Our findings link well to the complex in vivo situation and suggest that functional mucosal mimics can be created in situ for improved biomaterials performance.

Future perspectives

Clearly, the full biological effect of a biomaterial depends on many more factors than studied in the present work. Future work should involve studies on the interaction of mucin-coated materials with other components of the host defense system, primarily complement and platelets. Ultimately, whole blood experiments should be performed, to evaluate the combined effect of several host factors simultaneously.

Still, acting as starting point for future studies the present work is indeed promising. In this context, it should be mentioned that preliminary studies have indicated that mucin precoating could reduce the adsorption of complement (C1q and C3c) to a hydrophobic surface. Furthermore, although not included in this thesis, Paper VII demonstrated that PGM and MG1 coatings could be used to control the in-growth of human-derived epithelial, fibroblastic, and osteoblastic cells to an artificial material. Specifically, the strong inhibitory effect on fibroblastic cell adhesion suggested that mucin coatings could be used to reduce foreign body reactions at the tissue-biomaterial interface.

Common efforts during the last decade have greatly added to our knowledge about mucin structure and function. From a future perspective, such knowledge could contribute to the development of artificial coatings, which fully could mimick the normal mucus. One difficult task to solve is how to reproduce the complex glycosylation seen among the mucins [156]. Although there are many obstacles, promising examples on the synthetic design of mucin-like molecules exist [157,158]. One way to deal with the complexity of the mucin structure would be to employ recombinant protein technology. Thus, in one example it was shown that a genetically inserted MUC6 mucin fragment could confer stability and thereby protection to an enzyme [159]. Another interesting facet would be to explore the use of autologous mucins, extracted from the patient in question for ex situ coating of the implant. The present work tentatively support such strategy by showing that a human
salivary mucin, although being non-sterile and highly contaminated, greatly could reduce neutrophil adhesion and activation. Another level of sophistication would be to grow mucin-producing cells on biomaterial surfaces for continuous release of mucin to the implant surface. Mucin-producing cell lines have been available for some time in the pharmaceutical field, where they are used to estimate drug uptake and mucoadhesion [159].
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And now to the part of the thesis, which normally attracts most readers… There are of course many persons who have passed by over the years, of which everyone has a very unique meaning to me.

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My family, thank you for your love and support. Above all, I want to thank you for your enormous patience! I want you to know that this project not was intended as a study of inefficiency.
Utveckling av mucin-baserade biomaterialbeläggningar

Mucinerna utgör en komplext grupp stora, kolhydratrika proteiner som återfinns bundna på de flesta cellytor, samt lösliga i det tjocka, kladdiga sekret som utsöndras på kroppens slemhinnor (mukus). Länge har man ansett att mucinernas funktion huvudsakligen består i att agera fysiskt skydd åt slemhinnornas underliggande celler. Senare studier har dock visat att mucinerna inte bara är passiva barriärer utan faktiskt har aktiva roller i en mängd viktiga kroppsprocesser.

Mucinernas naturliga gränsskiktss funktion har fått oss att formulera hypotesen att dessa kan användas för generering av “kroppsegna” materialytor för användning inom biomaterialområdet. Tidigare studier från vår grupp har visat att ett salivmucin framrenat från ko (BSM) fördelaktigt kan användas för att minska proteiners, bakteriers och cellers bindning till plaster ofta använda i biomaterialapplikationer. Än mer intressant, en preliminär studie visade att ett plastmaterial belagt med BSM gav mycket låg immunologisk respons över 30 dagar i ett får. Specifikt, de vävnadsprover som analyserades visade att det mucin-belagda materialet haft väldigt låg inverkan på den kringliggande vävnaden.

Detta avhandlingsarbete bygger vidare på dessa resultat och har som mål att: 1) preparera väldefinierade muciner för användning som beläggningssubstanser, 2) utveckla metoder för analys av mucin-baserade ytbeläggningar, samt 3) utvärdera hur mucinbeläggningar påverkar mänskliga immuncellers och proteiners samspel med ett modellbiomaterial.

Arbetet börjar med att beskriva enkla procedurer för att rena ett mänskligt salivmucin (MG1) och fyra muciner från ko (BSM) och gris (PGM). Vi visar att det kommersiella BSM-startmaterialet varierar starkt i kvalitet mellan olika inköpta partier. Därför understryker vi vikten av att noggrant karakterisera muciner innan de används i jämförande studier. Av de renade mucinerna så är det humana mucinet komplext med andra komponenter, främst

Därefter visar vi att en vanlig använd metod för att bestämma proteinkoncentration på ytor kan kombineras med antikropps- och lektin-baserade metoder för att bestämma mängd och komposition för en komplekst ytbeläggning av mucin och ett annat protein. Av särskild vikt, presenteras en metod för att bestämma andelen exponerat protein i ett komplex mucinskikt.

Vi visar sedan att beläggningar med BSM, PGM och MG1 kan användas för att effektivt undertrycka responsen från neutrofiler, en viktig immuncellstyp, vid kontakt med ett modellbiomaterial. Genom att ändra förhållanden vid formuleringen av beläggningarna kan vi uppnå en liknande effekt för alla muciner. Detta indikerar att mucinerna liknar varandra med avseende på funktion.

Detaljerad analys med kvartsmikrovåg (QCM-D) föreslår att mucinbeläggningarnas förmåga att minska immuncell-aktivierung relaterar till deras förmåga att skära den underliggande ytan från kontakt med immuncellerna. Ett intressant delresultat var att den skärmande effekten var lika god för mucinbeläggningar som var tjocka och expanderade som för mucinbeläggningar som var tunna och kompakt.


Slutligen kunde vivisa att beläggning av ytan med mucin effektivt kan reducera neutrofil-responsen gentemot ytbudna, vanligtvis immunstimulerande proteiner såsom IgG, sIgA och fibrinogen. En detaljerad analys visade att graden av exponerat protein i de blandade mucin-protein-beläggningarna till stor del bestämde den immunologiska responsen. Trots detta samband var de absoluta mängderna av exponerade immuno-stimulerande proteiner höga. Kopplat till det låga immunologiska gensvaret föreslog detta att andra faktor-
rer än proteinernas exponeringsgrad påverkade den immunologiska respon-
sen. Kvartsmikrovågsanalys kunde ge en förklaringsgrund till detta genom att visa att mucinbeläggningen samverkar och stabiliserar ett av de studerade proteinen (IgG) på ytan.

Våra samlade resultat kopplar väl till den komplexa situationen vid slemhinnorna och föreslår att funktionella mukus-skikt kan skapas på konstgjorda material för att förbättra deras prestanda i kroppen.
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