Investigation of the surface adsorption and biotribological properties of mucins

Madsen, Jan Busk

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Investigation of the surface adsorption and biotribological properties of mucins

Ph.D. thesis

by

Jan Busk Madsen

July 2014
Preface

This thesis has been submitted to fulfill the requirements for obtaining a PhD degree at the Department of Mechanical Engineering, Section for Materials and Surface Engineering, Technical University of Denmark. The thesis is divided into two sections: a theoretical introduction and an experimental part containing the aim of the project as well as published papers and manuscripts to which I have contributed. The theoretical introduction provides background information for the studies that were performed. The experimental work presented by the six manuscripts was carried out primarily at two departments at the Technical University of Denmark. Biophysics and tribology studies were done at the Section for Materials and Surface Engineering under the supervision of Associate Professor Seunghwan Lee. Protein purification and biochemical analysis was carried out at the Enzyme and Protein Chemistry group, Department of systems Biology, under the supervision of Associate Professor Maher Abou Hachem. The aim of my studies was to elucidate the biophysical and biotribological properties of mucins as a model for biocompatible lubricants. The experimental work is presented in the following manuscripts:

(I)  “A Simplified Chromatographic Approach to Purify Commercially Available Bovine Submaxillary Mucins (BSM)” We established a rapid one column purification method for BSM purification.

(II) “Lubricating properties of bovine submaxillary mucin (BSM) films on a hydrophobic surface: Influence of impurities and contact scale” We investigate the differences in tribological properties of purified-, dialyzed- and “as received” BSM.

(III) “Interactions between bovine serum albumin and bovine submaxillary mucin in solution: A spectroscopic study” We investigate time dependent interactions between BSM and BSA.

(IV)  “Thermostability of bovine submaxillary mucin (BSM) in bulk solution and at a sliding interface” We investigate the high temperature tribological stability of BSM.

(V)  “Comparative studies of gastric and submaxillary mucins: Influence of pH on the conformation, surface adsorption and aqueous lubricating properties” We investigate the impact of pH on the properties of BSM and porcine gastric mucin (PGM).
“Proteolytic degradation of the terminal domains of bovine submaxillary mucin (BSM) and its impact on adsorption and lubrication at the hydrophobic surface” We investigate the impact of removing the terminal domains from BSM.

Manuscripts where I have contributed, but are beyond the scope of this thesis, can be found in the appendices.

This work could not have been done without the assistance of several people and I would like to take this opportunity to thank past and present group members. I would also like to thank the rest of the section for Materials and Surface Engineering and also the people at Enzyme and Protein Chemistry for providing an enjoyable working environment. In particular, I would like to thank my supervisor Seunghwan Lee for giving me the opportunity to perform the work contained within this thesis and guidance during the last three years; Associate Professor Maher Abou Hachem is thanked for expert guidance in protein purification and insightful suggestions to the biochemical analysis. Nikolaos Nikogeorgos is thanked for fruitful discussions and critical review of this thesis.

Last but definitely not least, I would like to thank Janne, my family and friends for always being there.

July, 2014

Jan Busk Madsen
Appendix 3: Feasibility of bovine submaxillary mucin (BSM) films as biomimetic coating for polymeric biomaterials

Appendix 4: Unique Non-Fouling Surface (NFS) Generated with a Commercial Bovine Submaxillary Mucin Films: Resistance to Nonspecific Adsorption of Proteins
Summary

Tribology is the study of friction, wear, adhesion and lubrication. Biotribology covers all aspects of tribology that are related to biological systems. Most organisms face tribological challenges where increased friction is often desirable, such as walking, gripping and lifting objects or adhering to a surface. However, in other instances the inverse properties are desirable. Mucins are found on epithelial surfaces throughout the body and are a key component of the mucus barrier. Here, they facilitate friction reduction, thus lowering the impact of physical abrasions, but they also act as a physical barrier that reduces adhesion to, and penetration of, the epithelial cell layer by bacteria. The composition of the mucin macromolecules includes hydrophobic globular terminal domains that are separated by heavily glycosylated (hydrophilic) central domains. The central domains carry an overall negative charge due to the oligosaccharides being capped by negatively charged species such as sialic acid or sulphate groups. Mucins display phenotypic diversion according to their expression site. This is most pronounced in the oligosaccharide composition of the central domains. The amphiphilic nature of mucins and their aqueous lubrication properties have led to them being proposed as possible biocompatible lubricants.

In this thesis, we investigate the biotribological properties of two commercially available mucins on the soft, elastomeric and hydrophobic surface of PDMS under different conditions. Due to the presence of a significant amount of non-mucin biomolecules in the commercial mucins, a mild single column protein purification protocol was established. In the mucin biotribology community, many employ the mucins either “as received” or after dialysis. It was therefore investigated how the established purification process impacts their adsorption- and tribological properties in comparison to either no purification or mildly purifying dialysis treatment. We show that the properties of the mucins are influenced by the presence of other biomolecules. Bovine serum albumin was determined to be the main protein contaminant in bovine submaxillary mucin (BSM). The interactions between these proteins were also investigated spectroscopically. Although these results are partly inconclusive, they hint that the mode of interaction between these proteins may be dependent on time and when the molecules are put into proximity to each other in solution. We have also investigated the thermostability of BSM. Most proteins denature at elevated temperatures. In this context, BSM displayed amazing resilience. PH was another environmental factor that was investigated. The results showed that the mucins surface adsorption and tribological properties is highly environment dependent. Lastly, the importance of the hydrophobic terminal domains in
surface adsorption and subsequent aqueous lubrication efficiency was investigated by proteolytic digestion. Our studies show that the hydrophobic terminal domains are integral for adsorption and efficient aqueous lubrication of hydrophobic surfaces.
**Resumé**


**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AEC</td>
<td>Anion exchange chromatography</td>
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<tr>
<td>arBSM</td>
<td>As received bovine submaxillary mucin</td>
</tr>
<tr>
<td>arPGM</td>
<td>As received porcine gastric mucin</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BSM</td>
<td>Bovine submaxillary mucin</td>
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<tr>
<td>CBB</td>
<td>Coomassie brilliant blue</td>
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<td>CD</td>
<td>Circular dichroism spectroscopy</td>
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<td>CV</td>
<td>Column volume</td>
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<td>FFAs</td>
<td>Free fatty acids</td>
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<td>FL</td>
<td>Fluorescence spectroscopy</td>
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<tr>
<td>d-BSM</td>
<td>Dialyzed bovine submaxillary mucin</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>Ethylene diamine tetraacetic acid</td>
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<td>EtOH</td>
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<td>GalNac</td>
<td>N-acetylgalactosamine</td>
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<tr>
<td>HCHA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
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<tr>
<td>MALDI-TOF MS</td>
<td>Matrix assisted laser desorption ionization time of flight mass spectrometry</td>
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<td>MGs</td>
<td>Monoglycerides</td>
</tr>
<tr>
<td>MPa</td>
<td>Megapascal</td>
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<tr>
<td>NFS</td>
<td>Non-fouling surface</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>NIBS</td>
<td>Non-invasive back scatter</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>OWLS</td>
<td>Optical waveguide lightmode spectroscopy</td>
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<tr>
<td>PAS</td>
<td>Periodic acid/Schiff</td>
</tr>
<tr>
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<td>Phosphate buffered saline</td>
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<tr>
<td>PGM</td>
<td>Porcine gastric mucin</td>
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<tr>
<td>PTS-domain</td>
<td>Proline, threonine and serine repeat domain</td>
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<tr>
<td>QCM-D</td>
<td>Quartz crystal microbalance with dissipation monitoring</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEA domain</td>
<td>Sperm protein, Enterokinase and Agrin domain</td>
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<td>Size exclusion chromatography</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
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<tr>
<td>TGs</td>
<td>Triglycerides</td>
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<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>VNTR</td>
<td>Variable number of tandem repeats</td>
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Introduction

Introduction to tribology

Tribology is a term in science and mechanical/materials engineering that deal with the interaction of surfaces in relative motion and include the study of friction, adhesion, lubrication and wear. The term was first coined by David Tabor and H. Peter Jost in 1966 and was published in what has come to be known as “The Jost report” as a consequence of recognizing the problems of increased friction and subsequent wear in machinery [1]. The report estimated that Great Britain could save £515 million per year (1965 values) by reducing production costs from a decrease in (among other expenditures) energy consumption, costs of lubricants and maintenance and replacement of machinery by application of friction and wear reducing technology [2]. It was also estimated that further savings could be achieved by investing in research in the field. Various scientific and engineering disciplines such as physics, chemistry and mechanical/materials engineering had research areas focusing on tribology studies prior to the publication of the Jost report. However, the interdisciplinary nature of tribology had not been recognized. A following addition to the multidiscipline of tribology has been studies in the biological field. This new area of study was termed biotribology in 1973 by Dowson and Wright [3]. A general description of areas of interest in biotribology can be found below.

Friction

Kinetic friction can be described as the force that resists the lateral movement of two surfaces that are in mechanical contact with each other. The relative motion of the two surfaces in contact causes kinetic energy to be converted into heat. The friction between two moving surfaces originates from interatomic and intermolecular forces where interfacial bonds are formed and broken between the two contacting surfaces. In so-called dry friction these movements may cause wear to occur on the surfaces. Historically, the first recordings of friction studies were done by Leonardo Da Vinci. However, Da Vinci never published his findings on dry friction and they were thus “re-discovered” by the French physicist Guillaume Amontons in 1699 who attributed friction to surface irregularities and the consequent amount of force required to lift one surface over the asperities of the other [4]. Amontons eventually formulated and published what has since come to be known as Amontons’ 1st and 2nd laws of friction:

1) The force of friction is directly proportional to the applied load.
2) The force of friction is independent of the apparent area of contact.
The understanding of the fundamentals of friction was not further developed until 86 years later when Charles-Augustin Coulomb formulated and published his thoughts on friction. Coulomb considered several more factors in the understanding of friction: the extent of the surface area, the length of time the surfaces remained in contact, the normal pressure and also the material that made up the contacting surfaces [4].

This led to the formulation of Coulomb’s law of friction which also came to be known as the 3rd law of friction:

**Kinetic friction is independent of the sliding velocity.**

In 1950, Phillip Bowden and David Tabor proposed a physical explanation for the observed behavior of friction. They argued that the true area of contact formed by asperities on the contacting surfaces is a very small percentage of the apparent area. By increasing the normal forces, more asperities come into contact and the average area of each contact asperity grows, indicating that the frictional forces are dependent of the true area of contact. Furthermore, it was also established that adhesive interactions between the contacting asperities play a significant part in the overall frictional force [4].

The friction force between two surfaces moving relative to each other can be defined as the force applied to a system in the opposite direction of the movement of the surface Figure 1. From this, the coefficient of friction ($\mu$) is defined as: $\mu = \frac{F_{friction}}{F_{normal}}$, where $F_{friction}$ is the friction force and $F_{normal}$ is the total force between the two surfaces.

![Figure 1 Schematic representation of the forces acting upon an object sliding on a surface in a dry contact environment.](image)

On a horizontal plain where no external load is applied, the only influence on the sliding body would be gravity and the coefficient of friction would therefore be: $\mu = \frac{F_{friction}}{mg}$, where $m$ is the mass of the sliding body and $g$ is gravity. Usually adhesion is not taken into account at the macroscale due to its very small magnitude relative to the mass of the sliding bodies. Amontons’ 1st
and 2nd laws seem directly applicable to such a system. The reason is that the real area of contact is not equal to the apparent area of contact. So, an increase in $F_{\text{normal}}$ (due to increased mass) can explain increased friction as a result of an increase in the real area of contact and a plastic deformation in the asperities. It has been demonstrated that the real area of contact can vary linearly with $F_{\text{normal}}$ when either multi-asperity contact occurs or plastic deformation takes place [5, 6]. Thus, the linear relation between friction and the $F_{\text{normal}}$ can be explained.

The three laws of friction are mainly applicable to dry friction systems as the overall properties of a system changes when for example a lubricant is added. Also, the advancement of technology has shown that these laws of “macro” friction are not necessarily applicable at the nanoscale.

**Lubrication**

As described in the first section, tribology deals with friction, wear and lubrication. Lubrication is an excellent way to reduce any wear that might occur on surfaces in close proximity. The application of lubricants is not a new concept [7]. A lubricant is a substance that interposes itself between the two sliding surfaces and can be either a solid material such as graphite, liquids such as different types of oil and in rare cases even gases have been used as lubricants. Here we will focus on aqueous lubrication as that is the scope of the work presented in this thesis.

Fluid lubrication is usually split into three regimes: boundary lubrication, elasto-hydrodynamic lubrication and hydrodynamic lubrication [8, 9]. The boundary of the three regimes of lubrication is not set and was first described by Richard Stribeck approximately 100 years ago and is still valid to this day [10]. Figure 2 shows a typical example of a Stribeck curve for a viscous lubricant between two sliding surfaces.
Hydrodynamic lubrication occurs in the high speed regime (Figure 2). Under hydrodynamic lubricating conditions, the sliding surfaces are completely separated by a fluid lubricant and the normal load is supported by the pressure within the fluid lubricant film. The pressure which supports the separation of the surface originates from the change in viscosity of the lubricant at high speeds. For hydrodynamic lubrication to occur, the geometry of the sliding surfaces must be slightly skewed causing them to converge. This is seen for instance in journal bearings. Further increasing the speed or decreasing the normal load in the hydrodynamic range causes the coefficient of friction to increase. This is due to the viscosity of the lubricant increasing as a function of the shear stress imposed on it causing a drag effect.

At intermediate velocities, the effect of elastohydrodynamic lubrication is observed (Figure 2). In the elastohydrodynamic range, the sliding surfaces are separated by a thinner lubricant film than in the hydrodynamic range. As a consequence (when the two contacting surfaces are non-conformal) the surface contact areas are usually very concentrated. This means that a lot of pressure is exerted onto a very small area. Due to the very high contact pressure (up to several GPa) between the surfaces, the viscosity of the lubricant and the deformation of the sliding interface play an important role in reducing the coefficient of friction. When the surfaces are made of soft elastic bodies such as rubber, only the deformations within the contact area are considered. This is known as soft EHL. When two elastic surfaces are pressed against each other, the contact area expands with increasing load. The elastic distortion of the surfaces generated by hydrodynamic pressure of the lubricant
causes an almost parallel thickness distribution of the lubricant layer. The exception is at the exit region of the contact area where the pressure increase deforms the contact surface further. At slow speeds or really high pressure/load the sliding interfaces of the two surfaces do not separate. This is the boundary lubrication regime where lubrication is dependent on the adsorption of lubricating molecules onto the surface (Figure 2). Repulsive forces between the adsorbed layers carry the load and prevent adhesion between contacting asperities thus lowering the overall friction between the surfaces.

As can be seen in Figure 2, a fourth region known as mixed lubrication, may also factor into the lubrication between two surfaces. In mixed lubrication, both surface adsorbed and lubricant film separation of the surfaces impact the coefficient of friction leading to a significant drop.

**Biotribology**

The term biotribology was first coined in 1973 by Dowson and Wright to cover “all aspects of tribology related to biological systems” [3]. Most organisms face tribological problems. In some instances, reduced friction is achieved where key examples are the synovial fluid in the joints and articular cartilage during movement, transport of foodstuffs in the digestive tract or maintaining the smooth motion of the eyelids over the eye by providing the necessary moisture. However, in other instances elevated friction is preferable such as walking or friction in automotive breaks. Nature, through many million years of evolution, has produced some remarkably capable tribological systems that include hydrodynamic-, wetting-, adhesive- and aerodynamic properties of natural surfaces [12]. The success of some of these systems (and subsequent elucidation of) has led to many adaptations in science and engineering that have made it into everyday products. However many areas of study within biotribology are still ongoing and include diverse areas such as:

- Tribology studies of saliva lubrication [13, 14]
- Denture wear [15, 16]
- Contact lenses and eye interaction – ocular tribology [17]
- Wear on artificial heart valves [18]
- Tribology of synovial joints and artificial replacements [19, 20]
- Wear on screws and plates in bone fracture repair [21]
- Tribology of salivary substitutes [22]
- Friction and slipperiness of clothing in contact with the skin [23-26]
As mentioned, a good example of tribology in nature is joint lubrication. Articular joints pose a very complex problem with a very elegant solution. Structural stability in the form of hard bones is necessary for eukaryotic organisms such as mammals to be able to move around and function. However, bone on bone contact in the joints results in rapid and extreme wear due to high surface friction. The problem was solved evolutionarily by softening the surface contact by the addition of a soft, pliable layer between the contacting surfaces (Figure 3). Articular cartilage is a complex material that exhibits a gradient in its composition transitioning from mostly collagen at the bone to a mixture of collagen and proteoglycans at the interface in the cavity between the surfaces that is filled with synovial fluid (Figure 3) [27].

![Figure 3 Schematic of a normal, healthy synovial joint. Figure from [27].](image)

The articular cartilage acts as the load bearing surface in the joint and exhibits low friction and wear, thus providing smooth motion between the moving bones [28]. A cavity filled with synovial fluid separates the surfaces in the joint. Synovial fluid is found not only between the contacting surfaces, but also in reservoirs in the upper layers of the cartilage layer. The synovial fluid is a blood plasma-like mixture of many biomolecules such as hyaluronan and phospholipids, but also proteins like lubricins and aggregans [29-33]. Lubricins are highly glycosylated proteins that contain mucin-like central domains [31, 34, 35]. The high concentration of posttranslational oligosaccharide modifications on the protein causes it to swell in solution and retain water at the
interface, thus effectively reducing friction during sliding by acting as a boundary lubricant. Figure 4 shows a schematic representation of the forces acting on the articular cartilage during sliding. Movement of the joint, when running for example, results in compressive forces from either bone to act on the articular cartilage surface. This causes the porous articular cartilage to compress and the synovial fluid within is expunged into the contact interface. Lubrication in the joint between surface contact asperities is achieved in two ways; during initial low speeds and load, boundary lubrication by surface adsorbed lubricin and other biomolecules occurs. As pressure and speed increases, the formation of a film by the synovial fluid and the plastic deformation of the surface asperities, move the lubrication into the soft elastohydrodynamic regime [36-38]. The overall result of the lubrication is a coefficient of friction in the synovial joint less than 0.001 [39-41]. When comparing with synthetic surface coatings such as Teflon ($\mu = 0.04$), the materials in the joints are superior by an order of magnitude.

![Figure 4 Schematic showing the forces acting on the articular cartilage during movement. Figure from [28].](image)

Currently the man-made water based lubricants and adhesives are inferior when compared to those found in nature. Natural systems are highly adapted and adaptable to the environment. Biotribology affords insight into the natural systems and adaptation or mimicking of natural systems may afford beneficial progress in the future for surface and materials engineering.
Mucins and the mucus matrix at the epithelial cell surface

Mucins are a family of large proteins, that are either secreted or cell surface tethered, and are a key component in the mucus gels that coat the epithelial lining. They act as a passive barrier that protects the underlying tissue from physical abrasion and bacterial infection [42, 43]. Depending on the function and origin, mucins are found in either monomeric non-polymerizing form or in complex formations through polymerization [42]. While cell surface tethered mucins are mostly found as either monomers [44] or in a heterodimeric formation [45], secreted mucins usually form polymerized complexes [46]. Secreted mucins are produced in specialized goblet cells that are incorporated into the epithelial lining or in submucosal glands (Figure 5) [47]. In the specialized cells, they are stored as preformed mucin in a dehydrated form within granules [47-49]. Secretion occurs either constitutively or as a response to extracellular challenges which ensures a rapid response during physical abrasion or bacterial adhesion [49].

![Figure 5 Modes of mucin secretion are represented in this schematic. The goblet cells with green granules secrete mucin directly from their embedded position in the epithelial layer while submucosal glands with red granules secrete mucins via pores in the surface layer. The mucus layer is represented as a green layer above the epithelial lining. The grey triangle shows the concentration of Ca\(^{2+}\) ions in the mucus layer in accordance with the density of the mucus layer.](image)

Evidence has shown that the mucins are highly ordered when stored within the granules [50-52]. Upon secretion and hydration the order changes drastically due to expansion/hydration and the gel matrix is formed. Recent studies suggest that the unpacking of the mucins is driven by a concentration reduction in Ca\(^{2+}\) ions over a gradient in the mucus matrix [50-53]. A high concentration of Ca\(^{2+}\) ions are found near the epithelial cell layer where the newly secreted mucins...
are densely packed and the concentration diminish gradually towards the surface of the mucus layer (Figure 5). A sharp increase in the pH through the mucus layer has also been proposed to be integral to the unfolding of the mucin macromolecules [51]. Polymerization of the mucin macromolecules occurs during unpacking which infers the formation of the dense and disordered mucus matrix. Mucins have been found to interact at relatively low concentrations where overlapping occurred in concentration ranges of 2-4 mg/mL under physiological conditions [54]. At even higher concentration the overlapping leads to aggregation and subsequently gelation due to non-specific interactions as displayed by an increase in the viscosity of the solution [55, 56]. This is the so-called “sol-gel transition” where the solubilized mucins start to form into a more solid gelated state. Mucins on their own, however, are not able to form mucus gels. A comparison between saliva and purified Muc5B saliva mucin showed that concentrations of Muc5B 10-20 times higher than that found in whole saliva were required to replicate the gel-forming properties of saliva [57]. Thus other factors present in saliva must play an intricate part in the formation of the naturally occurring mucus gels and the same conditions are most likely true for other gel-forming mucins. The function of the mucus layer in the immune response is not solely as an impassive barrier. Many other biomolecules such as proteins, ions and lipids have been found to be embedded in the mucus layer and are an essential part of mucus matrix. Lipids have been identified in both PGM [58] and BSM purifications [59] and may be an incorporated component in the mucus gels. In the pig gastric mucosa, a large abundance of free fatty acids and cholesterol were identified, and are suggested to shield the mucins from attack by oxygen radicals, thus playing a role in maintaining a healthy mucosal barrier on the stomach lining [58]. Many proteins have been identified to associate with mucins that have specific functions in regards to the mucus layer or the surrounding environment. One protein that has been identified to interact with mucin and appears to play a significant role in gel formation is the trefoil factor. Addition of trefoil factor resulted in a 10-fold increase in viscosity and elasticity thus substantially increasing gel formation [60]. Many other proteins associated with mucins are known to be a part of the innate immune response and form micellular complexes with mucins; among these are statherins [61], histatins [61], amylases[61, 62], proline rich proteins [61, 62], lysozyme [62], non-mucin glycoprotein 340 [63], lactoferrin [62], sIgA [62] and IgG fc binding protein [64, 65].

With the abundance of biomolecules reported to interact with mucins, it is not surprising that the mucins by themselves are not able to form mucus gels [42, 46]. Another factor in the investigation of the formation of mucus gels from mucins may be the purification method used. Using too harsh
conditions such as guanidium hydrochloride to solubilize mucus gels from tissue or scrapings may cause irreversible changes in the native structure of the mucins. Milder non-denaturing purification methods may therefore be preferable [59, 66]

**Expression of mucins**

Mucins are found at the epithelial lining throughout the body of all vertebrates. In humans, a total of 19 mucin genes are expressed and have been identified at the epithelial surface on a huge variety of organs and tissues. The mucins and their expression sites have been summarized in Table 1 where they have been categorized according to whether they are cell surface tethered or secreted. As previously mentioned, secreted mucins are synthesized in specialized mucin producing cells in submucosal glands or goblet cells embedded in the epithelial lining (Figure 5). Secreted mucins are found on highly diverse surfaces such as the respiratory tract, the eye, in the colon and the middle ear (Table 1). Biosynthesis of mucins is initiated in the endoplasmic reticulum where N-glycosylation also occurs. Furthermore, N-terminal dimerization and thus polymerization of the mucins is also initiated in the endoplasmic reticulum. After translocation to the Golgi apparatus, O-glycosylation modification of the apoprotein takes place [67-69]. As aforementioned, upon completion of the synthesis of the macromolecule, the mucins are stored in a densely packed conformation in granules within the mucin secreting cells until they are released onto the epithelial surface.

Cell tethered mucins are much smaller than their secreted counterparts and are usually made up of either a monomer [44] or two heterodimeric subunits [45]. For the heterodimers, the larger subunit usually resides outside of the cellular membrane and has structural similarity to the secreted mucins with heavy oligosaccharide modification. Cell tethered mucins are translated in a similar manner to the secreted mucins as a single peptide chain. During folding, the peptide of some of the cell surface tethered mucins undergoes autoproteolysis due to conformational stress within the SEA structural motif. Post cleavage, the two peptide subunits associate tightly [70-72]. O-glycosylation modifications are attached in the Golgi apparatus where the smaller subunit is also modified with palmitic acid allowing the mucin to be anchored into the plasma membrane of the epithelial cells [73, 74].
<table>
<thead>
<tr>
<th>Gene</th>
<th>Distribution</th>
<th>references</th>
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<tr>
<td><strong>Cell tethered mucins</strong></td>
<td></td>
<td></td>
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<tr>
<td>MUC1</td>
<td>Breast, gallbladder, stomach, cervix, pancreas, colon, duodenum, respiratory tract, kidney, eye, B cells, T cells, dendritic cells, kidney, middle ear epithelium</td>
<td>[75-81]</td>
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<td>MUC3A/B</td>
<td>Gall bladder, middle ear epithelium, duodenum, small intestine, colon</td>
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<td>MUC12</td>
<td>Uterus, prostate, kidney, stomach, pancreas, small intestine, stomach, colon</td>
<td>[88, 89]</td>
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<td>MUC13</td>
<td>Small intestine, colon, trachea, kidney, stomach, middle ear epithelium, appendix</td>
<td>[75, 89, 90]</td>
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<td>MUC15</td>
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<td>MUC16</td>
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<td>MUC17</td>
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<td>[75, 97]</td>
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<td>MUC20</td>
<td>Placenta, prostate, middle ear epithelium, liver, colon, kidney</td>
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<td>MUC5AC</td>
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<td>[75, 103-106]</td>
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<td>MUC6</td>
<td>middle ear epithelium, Gallbladder, pancreas, seminal fluid, stomach, cervix, duodenum</td>
<td>[75, 82, 110-112]</td>
</tr>
<tr>
<td>MUC7</td>
<td>Respiratory tract, middle ear epithelium, salivary glands</td>
<td>[75, 113, 114]</td>
</tr>
<tr>
<td>MUC19</td>
<td>Submandibular gland, respiratory tract, eye, middle ear epithelium, sublingual gland</td>
<td>[75, 115, 116]</td>
</tr>
</tbody>
</table>
The molecular structure of secreted mucins

As the work included in this thesis mainly focuses on the biophysical and tribological properties of secreted mucins, the scope of the following chapter will be a general description of the structure of secreted mucins while the structure of surface tethered mucins has been omitted. Mucins are large amphiphilic proteins where the major part of the macromolecule consists of one or several central domains that are heavily modified with mostly \( O \)-linked oligosaccharides (Figure 6) \[46, 69\]. Due to the native charge distribution of the carbohydrates, the central domains carry an overall negative charge at physiological conditions. The N- and C-terminal domains are usually described as being globular with an overall neutral charge due to a high content of uncharged amino acid residues. The primary structure of the apoprotein part of the central domains is made up of a variable number of tandem repeats (VNTR) consisting of proline-, serine- and threonine amino acid residues. Due to their compositions and presence in all mucins, they have been termed mucin like PTS-domains.

Oligosaccharides moieties are attached via the linkage carbohydrate N-acetylgalactosamine (GalNac) to the VNTR’s via the hydroxyl group of either serine or threonine (Figure 6). The abundant attachment of oligosaccharides to the central mucin-like domains increases the overall size of the macromolecule greatly by 2-3 times that of the naked apoprotein \[117\]. Oligosaccharides are reported to constitute between 50-80% of the total weight of the macromolecule, while the difference in the carbohydrate branching structures contributes to the diversity found in mucins. The most abundant oligosaccharides that are attached to the mucin apoprotein are either dimers or trimers of carbohydrates. However, even larger branched carbohydrate structures are interspersed throughout the central domain. Branched oligosaccharides containing up to six different carbohydrates have been identified to be present in bovine submaxillary mucin (BSM) \[118\] and porcine gastric mucin (PGM) \[119\]. Most of the smaller oligosaccharides are terminated by negatively charged groups such as the carboxyl group in sialic acid (pKa ~2.6) and sulphate groups (pKa ~1) rendering the overall charge distribution of the molecule to be highly negative under most physiological conditions. Analysis of BSM showed that up to 30% of the carbohydrates found on BSM are sialic acids \[120\] while up to 9% of the oligosaccharides found in PGM are sulphated \[121\]. The negative charge of the oligosaccharides is responsible for the intramolecular repulsion rendering the mucin to undertake their expanded “bottlebrush” like conformation depicted in Figure 6. An additional feature of the oligosaccharides is their ability to recruit water molecules from the surrounding environment due to the high concentration of hydrogen bonding groups on the surface thus conferring hydrophilic properties to the macromolecule. Cysteine rich so-called Cys domains
are found interspersed between the main mucin-like central domains (Figure 6). These domains are highly conserved both in the DNA and primary peptide sequence of many secreted mucins albeit their function has yet to be determined [122].

Figure 6 Conventional schematic representation of the secreted mucin. Globular domains are found in the N- and C-terminal regions of the macromolecule and are separated by heavily glycosylated central domains and Cys domains. The inset shows typical oligosaccharide structures found O-linked to the central domain of mucins. More information on the oligosaccharide structures can be found in [119, 123-125].

The N- and C-terminal domains of mucins are for the most part devoid of oligosaccharides apart from a few N-linked oligosaccharides attached at Asn-X-Ser/Thr acceptor sites in the primary peptide sequence [68]. The main function of the terminal domains is to take part in intermolecular interactions with other proteins, mainly bonding with other mucins [51, 67-69]. Common for all mucins is that the terminal domains are rich in cysteine residues. The cysteine rich domains termed D1, D2, D’ and D3 were first identified in the large multimeric glycoprotein known as the Von Willebrand factor (VWF) found in blood plasma where it mediates blood clotting by adhering to blood platelets and clotting factor VIII via disulfide formations [126]. Figure 7 shows a schematic representation of a typical distribution of D-domains in the terminal regions of secreted mucins. The occurrences of the VWF-like domains are not isolated to mucins and have thus been identified in several other proteins [127-130]. The D domains are believed to have a similar function in mucins as they do in the VWF, namely to facilitate the formation of disulfide linked polymerization between mucins [68, 69]. Recombinant expression of the terminal domain parts of mucin has
recently shown that the C-terminal domains usually form dimeric interactions with other mucins while the N-terminal domains form trimeric structures [51]. Other domains of note that are present in the terminal regions of some mucins are VWF-like B- and C domains and cysteine knots (CK) [46, 69]. Generally, the neutral charge of the terminal domains further promote interactions between them, but also facilitate surface adsorption *ex situ* on hydrophobic surfaces as well as interactions with non-mucin proteins [131-133].

![Diagram of mucin structure](image)

**Figure 7** Typical structure of a secreted mucin showing the domain distribution in the molecule. Below are shown the diversity found in the central domains of three different airway mucins. Figure adapted from [46].
Surface adhesion and aqueous boundary lubrication by mucins

Of the structural features of mucins that was described in the previous section, the hydrophobicity of the terminal domains have proven to be of paramount importance for the boundary lubrication properties of mucins between hydrophobic surfaces. Figure 8 shows the general consensus on how mucins adsorb onto the surface in a “dumbbell” like conformation with the hydrophobic terminal domains adsorbing onto the surface substrate and the central mucin like domains protruding away from the surface and into the solution. In the natural environment, however, the mucin like central domains may also play a role in adhesion to other biomolecules through either hydrogen bonding or electrostatic interactions [134].

Figure 8 Mucins adsorbed onto a surface with the hydrophobic “naked” terminal domains interacting with the surface and the central domains protruding into solution. Figure from [135]

Surface interaction and adhesion of mucins onto surfaces was initially investigated using the surface force apparatus (SFA) [136-138]. The advancement of other techniques such as atomic force microscopy (AFM) [139, 140], optical waveguide lightmode spectroscopy (OWLS) [131, 132] and ellipsometry [141] have subsequently been employed in determining the mass of surface adsorbed mucin, and in the case of AFM also their morphology. Mucins typically adsorb onto surfaces in the range between 2-5 mg/m² until saturation is achieved [142]. The mass adsorption is usually higher (though not always) onto hydrophobic surfaces than hydrophilic ones [143, 144]. AFM studies have suggested that the mucin molecules act more like rigid polymers than proteins in regard to their adsorption onto the surface (Figure 9).
The random coil structure of the mucin facilitates the possibility of several simultaneous contacts (i.e. mainly via the terminal domains) with the surface thus flattening the structure of the macromolecule onto the surface [146]. It was also determined via AFM that the mucins form a single molecule layer on the surface with a thickness of approximately 5 nm and that no further mass uptake occurs after the initial uptake [146]. *Ex vivo*, mucins and other similar biopolymers are interesting as they display excellent adhesion- and subsequent aqueous lubrication properties on hydrophobic compliant materials. The adsorption of mucins onto hydrophobic surfaces changes the outmost surface chemistry which can be determined easily, for example, by investigating the wettability of the surface through contact angle measurements [147]. Their conformation when adsorbed is also dependent on environmental conditions such as pH, ionic strength of the solution and surface chemistry [54, 136, 148, 149]. Furthermore, these factors may also impact the total adsorbed mass of mucins.

Tribological studies of mucins have been performed under many different conditions, at both macro- and micro scale, mainly using a pin-on-disc (PoD) tribometer, a mini traction machine (MTM), colloidal force microscopy or an SFA [131, 132, 138, 144, 150, 151]. Water on its own is generally a poor lubricant as its viscosity does not increase substantially with increasing pressure.
The addition of heavily glycosylated biopolymers such as mucin remedies this by recruiting water to the surface and thereby wetting it and introducing a low shear strength film between the two sliding surfaces [54]. The recruitment of water in the sliding interface acts as a lubricant due to water molecules being sheared away between the surface asperity contacts. However, the presence of the macromolecules themselves also introduces other factors that contribute to the reduction in friction such as intermolecular steric- and electrostatic repulsion forces [151-153]. Figure 10 shows the Striebeck curve for water and PGM at varying concentrations [153]. The addition of even very low concentrations of PGM in the solution (0.015%) shows a decrease in the coefficient of friction in the intermediate and high speeds regimes. Increasing the concentration of PGM to 0.1% and 0.2%, respectively, reduces the friction even further indicating that mucins do, in fact, act as a boundary lubricant in the low speed regime by adsorbing onto the surface.

![Figure 10 Striebeck curves for water and PGM at varying concentration using a silicone disc and steel ball tribopair, a temperature of 35°C and a load of 3 N. Figure from [153].](image)

As mentioned previously, environmental factors influence the adsorption properties of mucins in aqueous solution. This in turn also has an impact on the tribological properties of the macromolecules. Lee et al. investigated the effect of pH and ionic strength on the tribological properties of PGM when adsorbed onto hydrophobic PDMS [131]. They found that PGM was generally a poor lubricant at neutral pH, and an increase in the ionic strength of the solution only showed a slight improvement at the highest speeds (Figure 11). This indicated that PGM at neutral
pH adsorbed in a less than ideal amount onto the hydrophobic surface to support effective boundary lubrication.

Figure 11 Coefficient of friction plotted vs. speed for PGM in a pH 7 solution with varying concentrations of KCl. Figure from [131].

Figure 12 shows the result for PGM in solution at pH 2 with varying concentrations of salts added to the solution. Here they found that PGM is an excellent lubricant, whereas the concentration of added salt did not disrupt the surface adsorption at low and intermediate concentrations. At high salt concentration, however, the adsorption and subsequent lubrication was diminished as a consequence of the ion concentration in solution.

Figure 12 Coefficient of friction plotted vs. speed for PGM in a pH 2 solution with varying concentrations of KCl. Figure from [131].
Mucin adsorption under basic conditions was also investigated resulting in tribological properties similar to those under acidic conditions, and was ascribed to partial unfolding of the hydrophobic terminal domains facilitating increased anchoring onto the surface. The variation in lubricating properties according to pH and ionic strength indicate that mucins are highly adapted to their natural environment.

Other factors may also influence the lubricating properties of mucins such as surface roughness. In Figure 13, Stribeck curves from smooth and rough PDMS surfaces were compared. The results show that rougher surfaces, which have a higher number of asperities per area, reduce the effectiveness of mucins as a boundary lubricant. This is due to the higher number of asperity contacts per contact area. This is evident as the threshold speed where the coefficient of friction increases is higher for the rough surface tribopair when compared to the smooth surface tribopair. Only at very high concentrations of mucin (60 mg/mL) does mixed lubrication, at the intermediate speeds start to effectively lubricate (Figure 13b).

![Stribeck curves](image)

Figure 13 Stribeck curves of various mucin concentrations on smooth- (a) and rough surfaces (b). Figure from [151].

At the micro- and nanoscale, the method of choice has been the AFM and SFA [141, 146, 154-156]. Many studies have utilized hard surfaces such as mica or silica, that result in high contact pressures
(in the GPa range), very low loads and low sliding speeds that may be irrelevant for biological systems [139, 155]. However AFM affords the advantage to measure adhesion forces, wear and film formation thickness in the micro- and nanometer range [13, 157]. Studies of the tribological properties of mucin at the micro- and nanoscale are lacking at present. However, a study using a SFA with PGM as substrate on mica surfaces (Figure 14) [150]. Their results showed that PGM is an excellent lubricant up to the applied pressure of ~1MPa where the friction forces were barely registered. However, the coefficient of friction rose at even higher pressures (Figure 14) due to cross surface bridging and higher viscous dissipation at the interface.

Figure 14 Friction force measurements plotted as friction vs. load of mica surfaces coated with PGM. The results on the left were performed with hydrophilic surfaces, while the results on the right were performed on hydrophobic surfaces. The figures show results from different conditions after rinsing of the surface. Figures adapted from [150].

Mucins have been proposed a potential source of biocompatible coatings to reduce friction and wear [66, 133]. At present, though, the understanding of the tribological properties of mucins is lacking. The work presented in the following chapters seeks to add to that knowledge.
Aim of the current study

In classical engineering, oil based lubricants have been utilized for centuries to reduce friction and wear in mechanical systems such as ball bearing or machines. Even though oil based lubricants are excellent in reducing friction in mechanical systems, they have the significant drawback that they are sometimes toxic and almost always incompatible with biological systems. As mentioned in the introduction Dowson and Wright coined the term Biotribology in 1973 to cover the study of “all aspects of tribology related to biological systems”. Materials in nature show many astounding and inspiring properties such as sophistication, adaptability, miniaturization and wear resistance. Probably the most astounding property of them all is that evolution only allows for the selection of “just” good enough (and not perfection) to proliferate and become the dominant trait. Many natural systems show great potential as model systems for the advancement of micro- and nanotechnology in surface and materials science whereof mucins are one of them.

Mucins are the major macromolecular constituent of the mucous secretions that coat the respiratory, gastrointestinal- and urogenital tracts and many other surfaces in animals. The primary function of mucins and mucous gels is to provide protection to epithelial surfaces from invasive microbes and also physical insults by forming a lubricating layer. As described in the Introduction, mucins are highly complex amphiphilic molecules that are highly adaptive to their environment. Mucins have one major advantage as a lubricant over oil based lubricants and that is their biocompatibility. The general consensus of mucins is that they adsorb onto hydrophobic surfaces via their hydrophobic terminal domains while the central mucin like domains protrude away from the surface into the aqueous environment. Mucins act as lubricant by retaining water at the surface via hydrogen bonding and Van der Waals forces between the water and oligosaccharide chains of the central domains. In the boundary lubrication regime, the water will act as a thin film, and the shearing away of water molecules effectively lowers the shearing forces between two contacting surfaces. This inherent property of mucin has made it an obvious candidate for further studies, as either a biocompatible surface coating itself, or as a model for biomimetic synthetic polymers such as co-block polymers.

The motivation behind the work presented in this thesis is twofold: Firstly, mucins may serve as an excellent model for biomimetic design of synthetic polymers, such as brush forming co-block polymers that are able to both adsorb onto surfaces and retain water. Secondly, mucins may potentially be incorporated directly into the surfaces of existing biomedical devices. Coating of the
devices such as stents, catheters, laryngoscopes, endoscopes and even contact lenses could reduce tissue friction and subsequent damage or discomfort by usage. Thus it is of primary interest to better understand the fundamental adsorption and tribological properties of mucins.

The focus of the current project has been elucidation of biophysical and biotribological properties of mucins. We were interested in investigating how the mucins coped under certain environmental stresses as well as their response according to their origin. Other areas of interest were mucin interaction with other proteins and also changes in their adsorption and tribological properties after enzymatic treatment. To this end, commercially available bovine submaxillary mucin and porcine gastric mucin was employed. In the following chapters, the result of the work performed during this PhD project is presented in the form of either manuscripts or published peer reviewed papers. A short introduction has been added to each chapter explaining the motivation behind the work and my contribution to the specific project. Several manuscripts have also been added as appendices as they are beyond the scope of this project, i.e. studies of other mucin properties, or are mainly authored by another investigator.
Results and discussion

A Simplified Chromatographic Approach to Purify Commercially Available Bovine Submaxillary Mucins (BSM)

Commercially available mucins are known to contain other non-mucin proteins. In this study we established a simple one column anion exchange chromatography protocol for purification of BSM. The purification removed the majority of the non-mucinous proteins from commercial BSM. This was a crucial initial step that allowed for the analysis of the adsorption and tribological properties of BSM. The same protocol was subsequently employed to also purify PGM with slight modifications.

My contributions to this publication was establishing the column chromatography purification, impurity analysis by SDS-PAGE and MALDI-TOF MS, analysis of changes in hydrodynamic size by DLS and authoring the paper.
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A SIMPLIFIED CHROMATOGRAPHIC APPROACH TO PURIFY COMMERCIALY AVAILABLE BOVINE SUBMAXILLARY MUCINS (BSM)

Jan Busk Madsen a, Kirsi I. Pakkanen a, Lars Duelund b, Birte Svensson c, Maher Abou Hachem c & Seunghwan Lee a
a Department of Mechanical Engineering, Technical University of Denmark, Denmark
b MEMPHYS - Center for Biomembrane Physics, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Odense, Denmark
c Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, Denmark
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A Simplified Chromatographic Approach to Purify Commercially Available Bovine Submaxillary Mucins (BSM)

Jan Busk Madsen¹, Kirsi I. Pakkanen¹, Lars Duelund², Birte Svensson³, Maher Abou Hachem³, Seunghwan Lee¹

¹Department of Mechanical Engineering, Technical University of Denmark, Denmark
²MEMPHYS - Center for Biomembrane Physics, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Odense, Denmark
³Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, Denmark

Correspondence: E-mail seele@mek.dtu.dk, maha@bio.dtu.dk

Abstract

In this study, a simple purification protocol is developed to reduce the bovine serum albumin (BSA) content in commercially available bovine submaxillary mucin (BSM). This involved purification of the BSM by one-column anion exchange chromatography protocol resulting in BSM with greatly reduced BSA content, homogeneously distributed size, and in a high yield of ~43% from BSM as received from the manufacturer. The purity and composition of commercially acquired BSM was assessed by SDS-PAGE and mass spectrometry, which verified that BSA is the most abundant non-mucinous protein component. The purification effect was evident from a significantly altered CD spectrum of BSM after anion exchange chromatography.

KEYWORDS: mucin, bovine submaxillary mucin (BSM), bovine serum albumin (BSA), purification, anion exchange chromatography (AEC)
INTRODUCTION

Mucus layers protect various epithelial surfaces of mammalian organs, including respiratory, cervical, ocular, and gastrointestinal tracts against foreign bodies or external insult by providing hydration, lubrication, and a physical barrier. Mucins are synthesized by epithelium cells or submucosal glands, and are either secreted through the cell membranes to form mucus gels or incorporated into the membranes to constitute the glycocalyx. While secreted mucins are mainly related to protective functions, membrane-bound mucins are known to also participate in cellular signal transduction. For bovine mucins, recent data mining of the genome revealed that of fifteen mucin genes identified in the genome, nine and six encode membrane-associated and secreted mucins, respectively. Common for all mucins are tandem protein sequence repeats of varying length designated as mucin-like PTS-domains as they consist mainly of amino acid residue repeats of proline, threonine, and serine (PTS). The PTS-domains are heavily glycosylated by mucin type O-glycosylation. The overall lack of secondary structural motifs allows the mucin to adopt a “bottle brush”-like conformation having globular N- and C-terminal domains connected by an elongated polypeptide chain.

Mucins are often purified from mucus gels or the glycocalyx for molecular-level studies. In some of these studies, mucins have been purified from tissue sources by the authors, whereas in many others, commercially available mucins were employed as received from the manufacturer. While the first approach is advantageous in terms of the quality control of mucin samples, variation in the sample quality across studies is a concern. In this sense, commercially available mucins may have the potential to be used
as a standard between different studies. However, this has not been practical to date due to a few critical drawbacks; firstly, commercially available mucins are not able to regenerate mucous gels by simply increasing the concentration in solution,[20,21] indicating that certain irreversible changes occurred to the mucins in the course of the manufacturers’ isolation and purification. Secondly, they are known to contain a significant amount of non-mucinous biomolecules, such as albumin, immunoglobulins, and salts.[22-24] Presently, it is not known whether these are an integral part of mucous gels or “impurities” introduced during the manufacturers’ purification process.

Regardless of its origin, the presence of non-mucinous components in mucin samples in an uncontrolled fashion is of utmost significance, especially when mucins are allowed to interact with other substances such as proteins,[24] cells,[24,25] bacteria[11,14] or polymers.[12,13,15,18,19] The “impurities” may participate, even preferentially, in the interactions and therefore could be a source for misinterpretation. Lastly, commercially available mucins suffer from batch-to-batch variation in their content of “contaminating” biomolecules[26] which adds to complicating the comparison between these studies. It was therefore pertinent to investigate the effectiveness of dialysis as it is a convenient and frequently employed method for purification of mucins.[27–30] Recent studies by Sandberg et al.[22–24] employing a two-step column chromatographic purification approach showed a significant improvement in mucin purity and it can be considered as a standard purification protocol for commercial mucins. In that study, it was also shown that the major “impurity” of commercially available bovine submaxillary mucin (BSM) is bovine serum albumin (BSA), representing up to 9% of the total mass of “as-received” BSM.[31]
A strong aggregation of albumin to mucin was previously verified by an increase in viscosity of mucin solutions upon interaction with albumin in bulk\cite{32} or on a surface.\cite{33}

In the present study, we present a simplified purification protocol optimized for removal of BSA from commercially available BSM. To this end, firstly, we have characterized the impurities in commercially available BSM by aid of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), and thin layer chromatography (TLC). Next, we have purified commercial BSM samples by a one-step anion exchange chromatography. Using a two column chromatography purification strategy similar to that proposed by Sandberg et al.\cite{22–24}, we have produced BSM in our laboratory that had a higher degree of purity compared to “as-received” BSM (denoted as “ar-BSM”) from the manufacturer. However, it was also observed that the yield of purified mucins was inadequate for our purposes (~2% of initial material loaded). We hypothesized that anion exchange chromatography alone may provide sufficient purity of mucin, since the isoelectric point of BSA (pI 4.7)\cite{26} is sufficiently different from that of BSM (pI ~2–3).\cite{11,34} This approach is expected to be particularly useful when the aim of purification was mainly to remove BSA from commercial BSM.

**EXPERIMENTAL**

**Mucins And Chemicals**

BSM (M3895-1 G, Type I-S, Lot. Nr. 039K7003V and Lot. Nr. 039K7003; Note that two different batches were employed for comparison of purification product) and all
Purification Of BSM

Chromatographic purification of BSM was carried out by modifying the strategy proposed by Sandberg et al.,\[22\] consisting of size exclusion chromatography and anion exchange chromatography in series. In the present study, a modified anion exchange chromatography (AEC) protocol was employed. Briefly, 250 mg of ar-BSM was dissolved in 10 mM Na-acetate, 1 mM EDTA, pH 5.0 on a nutating mixer overnight at 4 °C to a final concentration of 10 mg/mL. The BSM solution was clarified by filtration (5 µm hydrophilic polyethersulfone sterile filter; Pall Corporation, Cornwall, U.K.) removing aggregates. Proteins present in ar-BSM were fractionated according to their charge at 4 °C on a high load 53 mL 16/26 Q Sepharose high performance anion exchange column (GE Healthcare Life Sciences, Uppsala, Sweden) installed on an Åkta Avant chromatograph (GE Healthcare, Uppsala, Sweden) by elution with a multi-step gradient of a high salt elution buffer, 10 mM Na-acetate, 1 mM EDTA, 1.2 M NaCl, pH 5.0. 25 mL of 10 mg/mL sample was applied to the column at a flow rate of 2 mL/min and subsequently washed with 2 column volumes (CV) of 5% elution buffer (0.06 M NaCl) to remove any non-bound proteins. By increasing the content of elution buffer to 21% (0.252 M NaCl) over 1 CV at a flow rate of 3 mL/min, proteins displaying weak ionic interactions with the column were eluted. More tightly bound proteins were eluted by a linear increase in the elution buffer from 21% to 24% (corresponding to 0.252–0.288 M NaCl), over 4 CV at a flow rate of 2 mL/min, where most of the BSA was separated.
from BSM. The final segment in the gradient consisted of stepwise increase to 100% elution buffer, which was maintained for 2.4 CV to elute tightly bound proteins. Fractions containing protein were analyzed by SDS-PAGE and stained using coomassie brilliant blue (CBB) to visualize proteins or periodic acid/Schiff staining to identify fractions containing glycoproteins. The fractions containing BSM were pooled, dialyzed against milliQ-grade water (400:1 volume ratio) and freeze-dried to give purified BSM (denoted as “ae-BSM”). The ae-BSM was stored at −20 °C and desiccated prior to use. ar-BSM dissolved in milliQ grade water was also dialyzed against milliQ grade water at a volume ratio of 400:1 overnight followed by switching to fresh milliQ grade water for 4 h at the same ratio. The dialyzed BSM (designated “d-BSM”) was freeze-dried, stored at −20 °C, and desiccated prior to use. All steps for both procedures were performed at 4 °C to reduce the possibility of proteolytic degradation.

Matrix-Assisted-Laser-Desorption-Ionization Time-Of-Flight Mass Spectrometry Analysis (MALDI-TOF MS)

MALDI-TOF MS was employed to identify non-mucin proteins in ar-BSM. The bands, revealed by CBB staining on an SDS-PAGE loaded with ar-BSM, were subjected to in-gel trypsin digestion. Briefly, bands were excised and washed 10 min in 100 µL 40% EtOH to remove the dye. EtOH was decanted and 30 µL 100% acetonitrile was added to the gel fragments for approximately 10 min. Cysteines were reduced by incubation with 50 µL 10 mM DTT, 100 mM NH₄HCO₃ for 45 min at 56 °C. The DTT solution was decanted and cysteines were alkylated in 55 mM iodoacetamide, 100 mM NH₄HCO₃ for 30 min in the dark. The gel fragments were washed in 200 µL 50% acetonitrile for 10
min and dehydrated in 100 µL 100% acetonitrile. The acetonitrile was decanted from the gel fragments which were subsequently allowed to dry in air to ensure complete removal. 2 µL of 12.5 ng/µL trypsin (Promega, Madison, WI, USA) in a 25 mM NH₄HCO₃ was added and the sample was incubated on ice for 45 min. An additional 10 µL 25 mM NH₄HCO₃ was added for rehydration and the samples were incubated overnight at 37 °C. A supernatant aliquot (1 µL) was applied to the anchor chip target (Bruker-Daltonics, Bremen, Germany), covered by 1 µL matrix solution (0.5 µg/µL of α-cyano-4-hydroxycinnamic acid (HCHA), 90% acetonitrile, 0.1% TFA) and washed by addition of 2 µL TFA. Excess was removed after 30 s. MS spectra were obtained using an Ultraflex II MALDI-TOF MS mass spectrometer (Bruker-Daltonics) in auto-mode using flex control v3.0 (Bruker-Daltonics) and processed by flex analysis v3.0 (Bruker-Daltonics). Peptide mass maps were acquired in reflectron mode with 500 laser shots per spectrum. Spectra were externally calibrated using a tryptic digest of β-lactoglobulin (5 pmol/µL). The MS spectra were searched against the NCBInr database for mammalians (database nr. 56546546) using the MASCOT 2.0 software (http://www.matrixscience.com) integrated together with BioTools v3.1 (Bruker-Daltonics). Filtering of spectra was carried out for peaks corresponding to known fragments keratin and trypsin autocatalysis.

Thin Layer Chromatography (Tlc)

To investigate the presence of lipids in BSM, extracts of 2 mg of ar-BSM, d-BSM, and ae-BSM were used for TLC according to the Bligh and Dyer method.[27] Thin layer chromatography (TLC) was performed using 5 cm × 10 cm silica gel 60 TLC plates from Merck (VWR, Copenhagen, Denmark) and developed as previously described.[35] Briefly,
the development for nonpolar lipids consisted of a first step using a mixture of benzene: diethyl ether: ethanol: acetic acid (60:40:1:0.05, by vol.) to 5.5 cm from the origin. In the second step, the plate was developed to the top with a mixture of hexane: diethyl ether (96:4, by vol.). For polar lipids, the plate was pre-developed up to full length with a mixture of methylacetate: 1-propanol: chloroform: methanol: 0.25% KCl (25:25:25:10:9, by vol.). Then, samples were applied and the plate was developed to 6 cm from the origin in a mixture of methylacetate: 1-propanol: chloroform: methanol: 0.25% KCl (25:25:25:10:9, by vol.). Finally, it was developed to the top by a mixture of benzene: diethyl ether: ethanol: acetic acid (60:40:1:0.05, by vol.). The plates were dried, dipped in a 10% (w/v) copper(II) sulfate in 8% (w/v) phosphoric acid and charred in an oven (Digiheat, J.P. Selecta s.a., Barcelona, Spain) by raising the temperature to 180 °C. Compounds were identified by comparison with known standards.

**Circular Dichroism (Cd) Spectroscopy**

Circular dichroism spectra were acquired in a quartz cuvette with 1 mm path length (Hellma GmbH & Co. KG, Müllheim, Germany) using a Chirascan spectrophotometer (Applied Photophysics Ltd, Surrey, UK). Total sample concentration in the cuvette was 1 mg/mL (w/v) for both BSMs and BSM-BSA mixtures. All samples were measured in PBS buffer and the buffer background was subtracted from the data. Spectra were recorded from 280 to 195 nm with step size of 1 nm and bandwidth of 1 nm and near UV CD spectra were recorded from 400 to 260 nm with step size of 2 nm and bandwidth of 0.8 nm. The measurements were performed at room temperature (22 °C). The presented data are average of three independent measurements, each averaged of three scans.
Dynamic Light Scattering (DLS)

BSM samples were prepared in 0.22 µm-filtered PBS buffer and measured using a Malvern Zetasizer Nano ZS two angle particle and molecular size analyzer (Malvern Instruments, Worcestershire, UK). In this approach, non-invasive back scatter (NIBS) technique with the measurement angle of 173° was employed. The light source is a He-Ne laser at 633 nm and the temperature was set at 25 °C. Disposable cuvettes (PMMA, Plastibrand) were employed for DLS measurements. The concentrations of samples were 1 mg/mL. The mixed samples of ae-BSM and BSA with varying ratio were prepared approximately 2 h prior to the measurements. In one control experiment, however, a mixed sample was stored for at 4 °C for ca. 66 h prior to the measurements. Each sample was measured in triplicates. The Malvern Zetasizer software (Version 7.02) was used to analyze the obtained data and all DLS data were illustrated in the form of the intensity distribution of the hydrodynamic diameter (Dh).

RESULTS AND DISCUSSION

Purification Of BSM

BSM was purified by means of anion exchange chromatography (AEC). Mildly acidic conditions at pH 5.0 (close to the isoelectric point of BSA) were chosen to minimize the overall charge of BSA. Release of bound protein by increasing the NaCl concentration was monitored by peptide linkage absorbance at 214 nm (Figure 1), as the relative content of aromatic amino acid residues per mass is too low in mucins to allow reliable measurements at 280 nm. Similarly to a previous report,[22] BSA elutes prior to the main BSM fraction. Analysis of fractions by SDS-PAGE (Figure 2) covering most of the peaks
in the chromatogram showed that BSM eluted as one major peak after BSA. SDS-PAGE was employed to visualize selected fractions eluting at various points from the column. Furthermore, it was essential to investigate whether material was lost during removal of aggregates by sterile filtration. It was found that it did not particularly impact the protein band intensity, suggesting that no significant loss of sample occurred during filtration (Figure 2, lanes “AR” and “SF”). SDS-PAGE also confirmed that most of the contaminants were separated from BSM as they are present at significantly lower concentration when compared to ar-BSM (Figure 2, lane AR and pools I, II and III).

Thirty fractions of 2 mL from the purification were combined into pools I, II and III (Figures 1 and 2). A minor fraction of high molecular mass putative BSM co-eluted with other contaminants including some smaller amounts of BSA only after 100% elution buffer was applied (peaks IV and V in Figure 1, Lanes IV/A, IV/B and V in Figure 2A) indicating a possible strong interaction between these proteins. It is very intriguing, however, that these proteins were not visible in the PAS glycostaining (Figure 2A). To verify that the BSA content in the purified BSM pools had indeed been reduced, BSM from after purification was run on an SDS-PAGE (Figure 2B). 75 µg of ar-BSM- or ae-BSM sample from two independent purifications were loaded and showed that there is a consistent reduction in the content of contaminating BSA in the purified BSM (Figure 2B). The yield of the purified BSM according to this procedure was ~43% (mass_{ae-BSM, obtained}/mass_{ar-BSM, loaded} × 100%) from this particular purification batch. Pool III was chosen for further investigation in the present work, as the presence of contaminating BSA was low.
As mentioned in the Introduction, there are several problems with the use of commercially available BSM without further purification. Of notable concern is contamination of ar-BSM with several other proteins, and the variation in the level of these contaminants from batch to batch, as was evident when investigating the size distribution of ar-BSM by DLS (Figure 6A) illustrating the presence of aggregates in a large range of varying sizes. This inherently makes comparisons across studies more complex. On the other hand, despite successful purification of mucins to high purity using a two-step chromatographic purification method proposed by Sandberg et al. [22] appears to be improper when a large scale yield of purified BSM is preferred. Thus, the currently proposed simpler purification protocol could be suggested as a quick and efficient alternative, especially when the aim of purification is to primarily minimize the content of BSA from BSM and a recover a high yield of purified BSM.

**Analysis Of Protein Impurities: MALDI-TOF MS**

MALDI-TOF MS is a highly sensitive method for protein identification by peptide mapping. Coupled with in-gel trypsin digestion of the protein bands visualized in Figure 3, ar-BSM was analyzed to identify non-mucin proteins.

By searching against the mammalian genomic database using the MASCOT search engine, BSM was identified as a smear on the gel above 200 kDa (Figure 3A), indicating that some degradation of BSM had occurred. As BSM is known to have a molecular weight in the MDa range, degradation fragments are most likely a byproduct of the manufacturer’s purification procedure. BSA was identified as the major contaminant and
was found to have migrated to three distinct positions on the gel. Analyzed gel fragments from the smear between 97 kDa and 200 kDa (Figure 3A) was identified as BSA in a bound, co-migrating form with glucose regulated protein 78 (GRP78). BSA was also identified as a smear around 40 kDa (Figure 3A). The presence of BSA in ar-BSM has previously been reported.[22] Posttranslational modifications of proteins are known to increase the complexity of the obtained MALDI-TOF mass spectrum. Two proteins that migrated to distinct positions on the SDS-PAGE were not identified due to very low probability scores in the MASCOT program (indicated by asterisks in Figure 3A). Firstly, this could be due to the low sequence coverage of peptide fragments identified in the spectrum. However, it is also possible that other posttranslational modifications of this sample than glycosylation could be present, further lowering the overall sequence coverage, thus resulting in even lower sequence coverage due to the altered mass-to-charge ratios of the modified peptide fragments. It is also a possibility that they are not mammalian of origin and were therefore not identified when searching in the MASCOT program. As they appear to be removed from the BSM by SDS-PAGE analysis, they were not investigated further.

Analysis Of Lipid Impurities: TLC

TLC was used to determine if any lipid components were present. As shown in Figure 3C, the nonpolar lipid analysis showed the presence of triglycerides (TGs), free fatty acids (FFAs), and monoglycerides (MGs) in ae-BSM and d-BSM while the signals of these lipids was weaker for ar-BSM. No polar lipids were detectable in any of the BSM samples (data not shown). The stronger intensity of the nonpolar lipid signal in ae-BSM
may indicate that interactions between nonpolar lipids and ae-BSM occur or that they co-elute from the anion exchange column. As nonpolar lipids are already present, even after anion exchange chromatographic purification, it was not possible to investigate their influence on the properties of BSM. It has not previously been reported that lipids are an integrated component of mucous gels. We therefore speculate that the presence of lipids could be an artifact of the purification performed by the manufacturer.

Spectrophotometric analysis of the ratio between absorbance at 260 and 280 nm suggests that no major nucleotide contamination was present in ar-BSM samples (data not shown). Dialysis has been used in mucin studies as a method of reducing impurities. Figure 3B shows a comparison between ar-BSM and d-BSM taken immediately after dialysis. It shows that there is basically no purification benefit by performing dialysis alone, at least when protein contaminants are concerned.

Conformational Variation Of BSM By Impurities: CD Spectroscopy

The influence of purification on secondary and tertiary structure of BSM was investigated by CD spectroscopy (Figure 4). The far-UV CD spectra of the three types of BSM samples (Figure 4 (A)) revealed characteristics of random coil conformation and α-helical or β-sheet structural motifs were hardly detectable in their structure. The lack of secondary structure is in agreement with the current understanding that the heavily glycosylated PTS-domains dominate the structure of mucins. Even though more organized structural components could be present in the N- and C-terminal domains, they are not substantial enough to alter the CD spectra of BSM dominated by the PTS-
domains. Despite the lack of strong secondary structure signals, some differences were observed from all three types of BSM. The positive 220 nm band, a characteristic for random coil conformation, is clearly visible with ae-BSM, but most likely convoluted with \( \alpha \)-helical motifs of BSA present in d-BSM and ar-BSM. The negative band at ~200 nm is somewhat different for all three BSM samples. While the difference between ae-BSM and d-BSM is mainly the intensity only, the negative band of ar-BSM is clearly shifted to higher wavelengths by 2–3 nm compared to ae-BSM and d-BSM. As will be addressed below, this is related to the higher amount of BSA in ar-BSM, and consequent occurrence of \( \alpha \)-helical features from BSA. Even though BSA is also present in d-BSM, its magnitude is relatively lower (see Figure 3B), and it may explain the lack of similar wavelength shift in CD spectrum of d-BSM. It should be noted though that the ae-BSM and d-BSM in Figure 4A are purified from the corresponding batch of ar-BSM. Thus, the shift of the CD spectra of ar-BSM compared to ae-BSM and d-BSM is valid only when compared to the same batch of ar-BSM because of strong batch-to-batch variation. As mentioned above, commercial BSM samples as received from the manufacturer have varying content of “contaminants” thus far-UV CD spectra of ar-BSM samples revealed significant batch-to-batch variation (data not shown).

Near-UV CD spectra of ae-BSM, d-BSM and ar-BSM (Figure 4B) were also measured. Near-UV CD spectra arise from vibrations and reorientations of aromatic amino acid residues and disulfide bonds, and thus changes in these can be interpreted as protein aggregation or higher organization such as tertiary structure.\(^{40,43}\) As shown in Figure 4B, no measurable near-UV signals were detected from all three BSM samples, which is
consistent with that BSM contains few aromatic amino acids and has no tertiary structure.\textsuperscript{[44]}

To better understand the influence of impurities, in particular of BSA, on the secondary structure of BSM, far-UV spectroscopy measurements were carried out on mixed samples of ae-BSM and BSA. The far-UV CD spectrum of BSA (1 mg/mL) presents strong double negative bands at 221 and 209 nm and a positive band at 198-199 nm (Figure 5A). This is in good accordance with the expected spectral features for an \(\alpha\)-helix in globular proteins\textsuperscript{[40]} and the BSA secondary structure is estimated to contain approximately 60\% \(\alpha\)-helical structure.\textsuperscript{[45-47]} When ae-BSM and BSA were mixed at a ratio of 20:1 (~5\% of BSA), the far-UV CD spectrum shifted to higher wavelengths by 1 – 2 nm, and starts to overlap with that of ar-BSM (Figure 5B), especially in the region of 205 – 210 nm. The positive band at ~220 nm that was visible exclusively from ae-BSM also disappeared.

When the amount of BSA was further increased in the mixture of ae-BSM and BSA, for example at a 10:1 ratio (~9\% BSA), the far-UV CD spectrum clearly shifted to higher wavelengths in comparison with, not only that of ae-BSM, but also that of ar-BSM. A further increase of the BSA ratio, e.g., 33\% (ae-BSM:BSA = 2:1) resulted in even further resemblance of the far-UV spectrum with that of BSA (data not shown). Furthermore, as the concentration of BSA in the mixture of BSM-BSA increased, the double negative minima, representing the \(\alpha\)-helical structure of BSA, emerge more strongly (Figure 5B).

All together, these data strongly support that BSA constitutes the most prominent part of the non-mucin components of ar-BSM.
Size Distribution Of BSM: Dynamic Light Scattering (DLS)

Lastly, DLS was employed to determine the hydrated size of BSM samples and to elucidate how it changes with purification. To visualize the changes in the hydrated size of the BSM as a result of purification, it was pertinent to analyze the size distribution according to peak intensity and not by volume or number of molecules. This was due to the actual number of BSA molecules present being much greater than that of BSM, which would therefore skew the plot giving a misrepresentation of the data.

Commercially available BSM has previously been reported to contain varying amounts of other biomolecules than BSM. The size distribution of ar-BSM from different bottles of commercial BSM was investigated (Figure 6A). The hydrated sizes of the BSMs are apparently influenced greatly as the aggregates present were found to vary over a wide size range (10 – 3000 nm). Purification was found to eliminate this variation (Figure 6B). ar-BSM and d-BSM were observed to display two major peaks, whereas ae-BSM revealed the presence of a single peak only (Figure 6B). Among the two peaks for ar-BSM and d-BSM, the higher intensity peaks showed the maximum average sizes at 350 nm (ar-BSM) and 300 nm (d-BSM) in diameter, respectively, and the smaller intensity peaks with maxima at about 40 nm in average diameter for both BSMs. For ae-BSM, two samples purified from two different batches of commercial BSM were analyzed. They showed similar size distributions with average sizes of approximately 40 nm and 48 nm, respectively (Figure 6B). No BSM molecules larger than ca. 200 nm in diameter was observed. In addition, no larger species with average sizes of ca. 300 – 350 nm in diameter that were present in ar-BSM/d-BSM were observed from ae-BSM samples. It
was noted that the peak maxima and the distribution of the smaller peaks present in ar-
BSM and d-BSM correspond to those of the single peaks of the ae-BSMs (Figure 6B).
The origin of the larger species with the average diameter of 300 – 350 nm present only
in ar-BSM/d-BSM is presently unclear; it could be BSM molecules with intrinsically
higher molecular weight, but could also be aggregates of BSMs or of BSM and other
biomolecules, all of which were removed during AEC purification.

CONCLUSIONS
Commercially available BSM has been reported to have a batch-to-batch variable content
of non-mucinous biomolecules. The major contaminant has been identified as BSA. In
the present work, we have established a rapid and efficient one column chromatography
method that provides greatly reduced BSA content, fairly homogenous size distribution
(average of ca. 40 – 48 nm and no molecules larger than 200 nm in diameter), and a high
yield of BSM (~43% recovered mass of initial material). The introduction of the
additional purification step greatly thus increases the comparability from study to study
of the biophysical properties of commercially available BSM. At present, it is not known
whether the origin of BSA in the ar-BSM is contamination from blood during isolation,
or if albumin is an integral part of the secreted BSM. Since commercially isolated BSM is
collected from the submaxillary gland tissue in a process that can be assumed to involve
gland homogenization, it is likely that at least part of the albumin originates from blood.

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Figure 1. Chromatogram of BSM separated on a Q Sepharose HP anion exchange chromatography column. The column was equilibrated in 10 mM Na-acetate, 1 mM EDTA, pH 5.0 prior to loading of the BSM solution (62.5 mL of 4 mg/mL, total weight in 250 mg solution). Peptide bond absorbance at 214 nm (black) and % gradient (red) are plotted as a function of elution volume. Bound proteins were eluted by a multi-step increase in NaCl (% elution buffer). BSM was collected and pooled into three fractions, I, II, and III. The asterisk indicates the peak of BSA, the most abundant contaminant. Tightly bound BSM (peaks IV and V) eluted after increasing the elution buffer concentration to 100%.
Figure 2. (A) Selected elution fractions from pools I, II, and III visualized by SDS-PAGE. The gels were stained using CBB- and PAS-staining. Lanes indicated by “AR” and “SF” indicate ar-BSM before and after sterile filtration to remove aggregates. The lane marked by one asterisk contained the eluted BSA, while double asterisk indicates the fraction between the BSA and BSM peaks (see Figure 1). BSM does not migrate into the gels due to its high molecular weight and appears at the top of the gel. Fractions were pooled (indicated I, II, and III) according to their elution time. The unmarked lane between pools I and II contains a fraction pooled with fractions from a different BSM purification. The Lanes indicates IV/A, IV/B and V show fractions from the peaks that eluted after an increase to 100% elution buffer (IV and V, respectively, in Figure 1). (B) Coomassie stained SDS-PAGE of ar-BSM and two samples of ae-BSM from independently performed purifications. The loaded amount of protein was normalized to 75 µg. The lane containing ar-BSM has been indicated by AR, while the ae-BSM has been indicated by 1 and 2, respectively. Lane 1 contains the ae-BSM used in this study.
Figure 3. (A) SDS-PAGE showing bands excised for in-gel trypsin digestion and subsequent MALDI-TOF MS analysis. Fragments of BSM (> 200 kDa) migrated into the gel at the top. BSA was identified in three individual bands either as a dimer co-migrating with the protein GRP-78, as a monomer or a fragment. Proteins in two excised bands (indicated by *) gave no significant hits against the mammalian database and were not identified. (B) SDS-PAGE comparison between 75 µg of ar-BSM before (ar) and after dialysis (d). (C) TLC of nonpolar lipids in the three mucin forms. Lanes containing ae-BSM, d-BSM, and ar-BSM are indicated by ae, d, and ar, respectively. Triglycerides (TG), free fatty acids (FFA), cholesterol (Chol), and monoglycerides (MG) were used as standards for identification of nonpolar lipids. TLC suitable for polar lipids was also conducted, but none were detected.
Figure 4. (A) Far-UV CD spectra of ar-BSM (red), d-BSM (blue) and ae-BSM (black).

(B) Near-UV CD spectra of ar-BSM, d-BSM and ae-BSM.
Figure 5. Far-UV CD spectra of ar-BSM (red), d-BSM (blue), ae-BSM (black), BSA (magenta), ae-BSM-BSA mixture containing ca. 5% BSA (light green), ae-BSM-BSA mixture containing ca. 9% BSA (dark green). (A) Full spectra and (B) close-up of the same spectra (d-BSM and BSA spectra have been omitted for clarity).
Figure 6. (A) size distribution of ar-BSM from three independent bottles of commercially available BSM. The ar-BSM used for purification in this study is indicated in cyan while the ar-BSM used for the second purification is indicated in orange. The third ar-BSM shown is indicated in dark blue. (B) Size distribution by intensity of ar-BSM (red), d-BSM (blue), ae-BSM bottle 1 (black), and ae-BSM purified from bottle 2 (dashed black) as measured by dynamic light scattering.
Lubricating properties of bovine submaxillary mucin (BSM) films on a hydrophobic surface: Influence of impurities and contact scale

Many previous studies involving commercially available mucin have employed them either “as received” or with slight purification in the form of dialysis. In this paper we were interested in comparing the adsorption and tribological properties of BSM at three levels of purification. The manuscript is currently undergoing peer review to be published in the journal Colloids and Surfaces B: Biointerfaces.

My contribution to this manuscript was supplying purified BSM, SDS-PAGE analysis, OWLS adsorption measurements and 1/3 of the PoD tribology measurements.
Influence of impurities and contact scale on the lubricating properties of bovine submaxillary mucin (BSM) films on a hydrophobic surface

Nikolaos Nikogeorgos, Jan Busk Madsen, and Seunghwan Lee*

Department of Mechanical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

*Corresponding author: seele@mek.dtu.dk

Key words: BSM, BSA, lubrication
Abstract

Lubricating properties of bovine submaxillary mucin (BSM) on a compliant, hydrophobic surface were studied as influenced by impurities, in particular bovine serum albumin (BSA), at macro and nanoscale contacts by means of pin-on-disc tribometry and friction force microscopy (FFM), respectively. At both contact scales, the purity of BSM and the presence of BSA were quantitatively discriminated. The presence of BSA was responsible for higher frictional forces observed from BSM samples containing relatively larger amount of BSA. But, the mechanisms contributing to higher friction forces by BSA were different at different contact scales. At the macroscale contact, higher friction forces were caused by faster and dominant adsorption of BSA into contacting area in the presence of continuous cycle of desorption and re-adsorption of the macromolecules from tribostress. Nevertheless, all BSMs lowered the interfacial friction forces due to large contact area and a large number of BSM molecules in the contact area. At the nanoscale contact, however, no significant desorption of the macromolecules is expected in tribological contacts because of too small contact area and extremely small number of BSM molecules involved in the contact area. Instead, increasingly higher friction forces with increasing amount of BSA in BSM layer is attributed to higher stiffness and viscosity caused by BSA in the layer. High contact pressure allowed AFM probes to penetrate through the BSM layers and scratch on the underlying substrates, and thus induced higher friction forces compared to the sliding contact on bare substrates.
1. Introduction

Epithelial tissue surfaces of human and animals, such as the respiratory, gastrointestinal, and reproductive tracts, are covered with viscous mucus gels that are formed from secretion of mucins by goblet cells or glands [1,2]. The main function of mucus layers is the protection of underlying tissues from physical insult and microbial infection. Lubrication is another characteristic of mucus gels, and the lubricating properties of saliva [3,4] and other mucus [5] have drawn significant scientific interest. Tribological properties of mucins at interfaces of synthetic materials have also long been studied [6-11] for its significance in various bioengineering applications, including saliva substitutes, contact lenses, food attributes, and condoms etc.

 Fundamental understanding of the lubrication mechanisms demands well-defined systems. While some biophysical studies have employed mucins that are isolated/purified from tissue sources by the authors [12-14], tribological studies have predominantly employed commercially available mucins as received from a manufacturer [6,7,9,10], or only after simple dialysis [8,15]. Despite its convenience, commercial mucins display several drawbacks, such as inability to form gels [16,17], the presence of “impurities” [17-19], and batch-to-batch variation in the magnitude of impurities. Particularly concerning for the tribological researches of mucins is the high affinity of non-mucinous impurities, e.g. proteins, lipids, and salts, to many materials surfaces [15,20,21], as well as mucins [22], and consequent impact on the adsorption and lubricating properties. In this context, recent attempts to further purify commercially available mucins by chromatography [18,23] are expected to provide an opportunity to investigate the lubricating properties of mucins with minimal influence from impurities.

This study focuses on the characterization of the tribological properties of BSM as affected by impurities. BSM from the same commercial batch was employed either as received (denoted as “ar-BSM”), after mild purification by dialysis (“d-BSM”), or after extensive purification by
chromatography ("ae-BSM") [23]. The tribological properties of BSMs were examined at macroscale and nanoscale contacts with pin-on-disk tribometry and FFM, respectively. A compliant and hydrophobic material, poly(dimethylsiloxane) (PDMS), was employed as substrate of the tribopair, while sliders with different size and chemistry have been employed, such as PDMS pins, Si$_3$N$_4$ pins, Si$_3$N$_4$ AFM probes, and octadecyltrichlorosilane (OTS)-modified AFM probes. The tribological properties of BSM are discussed in link with the relative size in bulk solution, magnitude of impurities, and adsorbed masses on the substrate surface.

2. Materials and Methods

2.1 Mucins and chemicals

BSM (M3895-1 G, Type I-S, batch 039K7003V) and bovine serum albumin (BSA, A7906-10G) were purchased from Sigma Aldrich (St. Louis, MO). All chemicals for buffers were laboratory grade and purchased from Sigma Aldrich.

2.2 Purification of BSM and SDS-PAGE

ae-BSM was prepared by means of anion exchange chromatography. Full description on the purification methodology are available in literature [23]. Briefly, ar-BSM was dissolved in Na-acetate buffer (10 mg/mL, 10 mM Na-acetate, 1 mM EDTA, pH 5.0), filtered through a 5 µm sterile filter (Pall Corporation, Cornwall, U.K.), and fractionated on a High Load 16/26 Q Sepharose High Performance anion exchange column (GE Healthcare Life Sciences). The fractions containing BSM were pooled, dialyzed against water, and freeze-dried, and kept in freezer (-20 °C) until use. Fractions were analyzed by SDS-PAGE, and Coomassie Brilliant Blue (CBB) was used to visualize protein content. All steps were performed at 4 °C. d-BSM was prepared by dissolving ar-BSM in MilliQ grade water, and dialyzing against MilliQ grade water (volume ratio, 400:1) overnight, followed by switching to fresh MilliQ grade water for 4 h at the same ratio.
2.3 **Dynamic light scattering (DLS)**

The hydrodynamic sizes of BSMs were estimated by DLS using a Zetasizer NanoZS (model ZEN3600, Malvern Instruments, UK). In this model, non-invasive back scatter technology (NIBS, measurement angle 173 °) was incorporated. The light source is a He-Ne laser at 633 nm and temperature was set at 25 °C. Disposable cuvettes (PMMA, Plastibrand®) were employed.

2.4 **Optical waveguide lightmode spectroscopy (OWLS)**

OWLS is a non-label technique to characterize the adsorption of molecules from solution to the interfacing solid surface. The waveguides (200-nm thick Si_{0.25}Ti_{0.75}O_{2} layer on 1-mm thick AF 45 glass) in this work were spin-coated with an ultrathin layer of polystyrene (6 mg/mL in toluene) at 2500 rpm for 15 s, and subsequently with an ultrathin (ca. 16 nm [24]) layer of PDMS at 2000 rpm for 25 s, and cured in an oven at 70 °C overnight. For PDMS, the base and curing agent of a commercial silicone elastomer (Sylgard 184 elastomer kit, Dow Corning, Midland, MI) were dissolved in hexane at a ratio of 10:3 (final concentration, 0.5 % w/w). OWLS experiments were carried out using an OWLS 210 Label-free Biosensor system (Microvacuum Ltd, Budapest, Hungary). The PS/PDMS-coated waveguide was first exposed to PBS buffer until a stable baseline was obtained. A programmable syringe pump was employed to transport buffer solutions through flow-cell containing OWLS waveguide surface. 100 µL of BSM sample was then injected via loading loop. Upon observing an increase in surface adsorption signal, the pump was stopped for 10 min. After rinsing the flow cell with PBS buffer, the adsorbed mass density data were calculated according to de Feijter’s equations [25]. The refractive index increment (dn/dc) value for BSM was determined to be 0.150 cm³/g.
2.5 Pin-on-Disc tribometry and tribopair

Macroscale tribological properties of BSMs were characterized with pin-on-disc tribometer (CSM, Peseux, Switzerland). Details on the operational principles of this technique are provided in previous studies [7,24]. The friction forces in this study were measured as a function of number of rotations (20) over a fixed track (radius: 6 mm) and a fixed load (1 N) while varying the speed of rotation. An average coefficient of friction, \(\mu\) (= friction force/load), for each speed could then be plotted. For the experiments with a layer of BSM coating, the number of rotation was extended to ca. 100. PDMS tribopair used in this study were prepared with Sylgard 184 elastomer kit mentioned above. Details on the process are provided in previous studies [7,24]. The dimension of PDMS disk was 30 mm \(\times\) 5 mm, and the pin radius was 3 mm. The same PDMS kit and the protocol were employed to prepare for “PDMS slab” with a thickness of 1 – 2 mm for AFM experiments. In order to remove un-crosslinked monomers, the slabs were immersed in toluene for 2 days, sonicated for 1 h in acetone and in ethanol. The Si$_3$N$_4$ pins (6 mm in diameter) were piranha-cleaned and thoroughly rinsed with ultrapure water prior to experiments.

2.6 Friction Force Microscopy (FFM)

FFM experiments were performed with a Digital Instruments Nanoscope III Multimode instrument (Bruker Instruments, Santa Barbara, CA), using a “J” scanner and V-shaped silicon nitride AFM probes (model NP, Veeco Instruments, Santa Barbara, CA) with a normal force constant of 0.24 N/m. All measurements were performed in liquids using a liquid cell (Veeco Instruments, Santa Barbara, CA). The normal spring constant of the cantilevers was characterized according to the generalized Sader method [26,27,28]. The normal photodetector sensitivity (nm\(\cdot\)V$^{-1}$) was acquired from force curves obtained on the flat regions of a silica specimen. Calibration of lateral forces was achieved using the ‘wedge calibration method’ [29,30], with a silica specimen.
prepared by means of focused ion beam milling [31]. The radius of curvature of the tip was obtained with the blind tip reconstruction method using a TGT01 grating (MikroMasch, Germany) [32].

For friction measurements, the instrument was operated in contact mode with the long cantilever axis perpendicular to the fast scanning direction (3 Hz scanning speed), over lines of 1 μm length, with the slow scan axis being disabled. Friction forces were determined from trace – retrace friction loops [33,34]. All bare probes were cleaned using piranha solution prior to use. *Piranha solution has very strong oxidizing power and is extremely dangerous, and thus should be handled with extreme caution.* The probes were subsequently rinsed with copious amounts of ultrapure water, dried in a flow of nitrogen, and used immediately. Silanized probes were prepared using OTS (> 90%, Sigma Aldrich, St Louis, MO); after the probes had been piranha-cleaned, rinsed with water, thoroughly dried, then immersed in a solution of 20 mL decahydronaphthalene (Merck, > 99%) with 2 drops of chloroform (Alfa Aesar, HPLC grade) and 2 drops of OTS for 30 min. They were subsequently rinsed with cyclohexane (VWR, > 90%), then with ethanol, and dried with N₂ flow.

3. Results and Discussion

3.1 SDS-PAGE: Impurities in BSM

The SDS-PAGE of BSM samples are presented in Figure 1. The strong bands with molecular weight higher than 200 kDa can be assigned to BSM or its fragments from their high molecular weights. Furthermore, they were the only bands that were detected by Periodic acid-Schiff (PAS) staining, and moreover, were identified as BSM with MALDI-TOF mass spectrometry in a previous study [23]. Various smaller molecular species other than BSM are present in ar-BSM, where the most clearly identified impurity is BSA (m.w. ca. 66 kDa). From d-BSM, slightly weaker band
strength for BSA compared to ar-BSM was observed, whereas even smaller species with the molecular weight < ca. 20 kDa were mostly removed. BSA and other smaller species were nearly not detectable from ae-BSM.

3.2 DLS: Size distribution

Representative plots for the hydrodynamic diameter ($D_h$) of different BSMs are presented in Figure 2. $D_h$ is plotted according to peak intensity, not by volume or number of molecules, to avoid misrepresentation of the data due to the size difference in BSM and BSA. ar-BSM and d-BSM presented two major peaks, whereas ae-BSM revealed a single peak. Among the two peaks for ar-BSM and d-BSM, the peaks for smaller species revealed maxima at about 50 nm and 38 nm in $D_h$ (“AR-I” and “D-I”), whereas the peaks for larger species showed the maximum $D_h$ at ca. 400 nm (ar-BSM) and 310 nm (d-BSM) in $D_h$ (“AR-II” and “D-II”), respectively. For ae-BSM, only a single peak distribution with the maximum at 43 nm (“AE”) is visible, and this peak is comparable to AR-I and D-I. Thus, in addition to the removal of impurities, additional effect of chromatographic purification of ar-BSM in this study is to remove large species with a $D_h$ of > ca. 200 nm. As larger species tend to scatter light stronger, AR-II and D-II peaks in ar-BSM and d-BSM should not be taken to reflect the number population of these species. The exact origin of the larger species in ar-BSM and d-BSM is unclear. These could be BSM molecules with intrinsically higher molecular weight, but could also be aggregates of BSMs or of BSM with other biomolecules. The distribution of $D_h$ for ar-BSM is fairly different from those previously reported in literature [35,36], probably due to batch-to-batch variation in the magnitude of contaminants in commercial mucins [18,23].

Since the major compositional difference between ae-BSM vs ar-BSM/d-BSM is BSA [23], and only ae-BSM shows the lack of large species (AR-II and D-II in Figure 2), it is of interest
whether BSA acts as linker between BSM molecules. The BSA and the mixtures of ae-BSM/BSA samples at the ratio of 20:1 were studied with DLS. The ratio of 20:1 (v/v) was chosen based on an estimation of BSA amount in ar-BSM in a previous study [20]. Upon mixing ae-BSM and BSA, the distribution of $D_h$ (Figure 2) showed no clear increase in size compared to ae-BSM alone. Instead, a small shoulder was clearly observed in the ae-BSM/BSA. The average size of this smaller peak was 7 nm (Figure 2), which corresponds to the size of BSA [37]. Hence, the addition of BSA to ae-BSM at the ratio of 20:1 (ae-BSM/BSA) did not induce immediate aggregation between them.

3.3 OWLS: Adsorption of BSM onto PDMS surfaces

Figure 3 shows representative surface adsorption profiles of the BSM samples as a function of time. For comparison, a representative surface adsorption profile of BSA is also presented. Average adsorbed masses of each type of BSM and BSA were obtained from 3-5 measurements. Similar adsorbed masses for ar-BSM and d-BSM were obtained, 189 ± 6.1 and 182 ± 32.8 ng/cm$^2$, respectively, whereas a significantly lower adsorbed mass, 49 ± 6.1 ng/cm$^2$ was observed from ae-BSM. The adsorbed mass for BSA was 43 ± 21 ng/cm$^2$. At first, it is tempting to attribute higher adsorbed masses measured from ar-BSM and d-BSM to commonly present larger species in them (AR-II and D-II in Figure 2). However, as was already mentioned above, the number distribution of these large species is, in fact, very small. Moreover, since adsorption of macromolecules onto surfaces is initially diffusion-controlled, smaller species are likely to adsorb faster than larger ones [15,38]; the adsorption time is 10 min only, and higher adsorption of ar-BSM and d-BSM is evident from the very initial stage of adsorption process. Thus, higher adsorbed masses from ar-BSM and d-BSM can rather be attributed to the abundant smaller non-mucin species present including BSA and other proteins (Figure 1).
3.3 Macroscale lubricating properties of BSM

3.3.1 Lubricating properties of ar-, d- and ae-BSM

Figure 4 shows the $\mu$-vs-speed plots obtained from different BSM solutions with pin-on-disk tribometry. Compared to the buffer reference, $\mu$ values have decreased by 1-2 orders of magnitude in all three BSM solutions in the entire range of speeds examined. As has been well-established from previous studies [7,11,40,41], high frictional forces of self-mated sliding contacts between PDMS surfaces in aqueous environment are ascribed to the strong interfacial hydrophobic interaction. Lubricin [42,43] or synthetic macromolecules [40,41,44] displaying amphiphilic characteristics with highly hydrating moieties can adsorb onto the surface, suppress the hydrophobic interaction, and effectively lubricate the sliding contacts between hydrophobic surfaces. In particular, once the hydrophobic interaction is suppressed, tribological contacts of PDMS/PDMS can be readily lubricated with aqueous solution for its high compliance and consequent low contact pressure. BSM is an ideal additive in this context, since its unglycosylated C- and N-termini are known to function as anchoring groups in the adsorption onto hydrophobic surfaces [15]. Moreover, highly hydrophilic glycosylated region of BSM can recruit water and assist to form aqueous lubricating films.

Despite the generally effective lubrication of all BSM solutions, the $\mu$ values were noticeably different for each type of BSM. For instance, the $\mu$ values of ae-BSM were measured to be lowest, e.g. $\mu = 0.003$ at the lowest speed, whereas those of ar-BSM were clearly higher, varying between 0.04 and 0.09. d-BSM showed intermediate $\mu$ values ranging from 0.007 to 0.019, suggesting that BSM becomes more slippery with increasing purity. Interestingly, the $\mu$ values of d-BSM are closer to those of ae-BSM, although its adsorbed masses are closer to those of ar-BSM. Thus, the lubricating efficacy of different BSM solutions is not linearly correlated with the surface adsorbed...
mass. In fact, ae-BSM showed the smallest adsorbed mass, but the most effective lubricating capabilities. This discrepancy can be explained by that surface adsorption of ar-BSM is contributed by these small species to a large extent that the resulting lubricating capabilities are less optimal than those of ae-BSM layer. While BSM is heavily glycosylated in the central Proline Threonine Serine (PTS) domain, and thus facile hydration can be achieved as mentioned above, other molecules in ar-BSM, including BSA, lack such structural features. Even though dialysis does not remove all the impurities from ar-BSM, the weaker band signals for non-mucin species from d-BSM in SDS-PAGE (Figure 1) suggest that the amount of impurities is indeed reduced, hence improving the lubricating properties of d-BSM.

3.3.2 Role of BSA in the lubricating properties of BSM solutions

Previous studies have shown that various other biophysical properties of commercial mucins than lubrication, such as viscoelasticity and surface forces, are also greatly changed after chromatographic purification, and the differences were attributed to the presence of BSA in as-received BSM samples [18,22,20,39]. Thus, tribological properties of BSA (1 mg/mL in PBS), as well as a mixture of ae-BSM/BSA (20/1 v/v) to simulate ar-BSM [20] have also been characterized. The results are presented in Figure 4. The $\mu$ values of BSA solution were much higher than those of BSM solutions, particularly in low-speed regime. In other words, intrinsic boundary lubricating efficacy of BSA is substantially inferior to that of BSM, and it can partly account for the relatively higher frictional forces of BSM with higher amounts of BSA, such as ar-BSM.

However, it should be noted that tribological properties of ar-BSM are not reproduced by ae-BSM/BSA mixture; ae-BSM/BSA showed a decreasing $\mu$ values with increasing speed, whereas ar-BSM showed a slightly increasing $\mu$ values with increasing speed. In fact, the $\mu$-vs-speed plot of ae-BSM/BSA rather resembles that of BSA. In other words, the tribological properties of the ae-
BSM/BSA mixture appear to be dominated by BSA. In pin-on-disc tribometer contacts, repeating cycles of tribostress can induce continuous desorption and readsorption of surface-adsorbing species from the solution [45]. Thus, for each revolution of the pin on the disc, surface adsorption of BSM and other molecules should be continuously re-established. In this process, fast-diffusing lighter molecules can dominate the surface adsorption and tribological properties of the contacting interface. The difference between ae-BSM/BSA mixture and ar-BSM can be explained by the abundance of “free” BSA in the mixture of ae-BSM/BSA, as shown in Figure 2. The absence of the peak for BSA in the DLS data of ar-BSM (Figure 2) is thought to arise from that a majority of BSA molecules are integrated into the aggregates formed with BSM.

In order to verify the “self-healing” mechanism proposed above, the lubricating properties of a layer of BSM coating were characterized and compared with those of BSM solution. To this end, ar-BSM was employed. After friction forces were acquired over a longer number of revolutions (ca. 100) under a fixed load and speed in ar-BSM solution, the PDMS disc was rinsed with PBS buffer. Then, the same experiment was performed in PBS buffer (data shown in Fig S1, Supplementary Data). As expected, low $\mu$ values (ca. 0.024) were maintained for the entire duration of experiments in BSM solution. But, in PBS buffer with a layer of ar-BSM coating on the PDMS disc surface, the initial $\mu$ of 0.047 gradually increased to 0.54 within 100 revolutions. This is most likely due to gradual, yet irreversible removal of ar-BSM layers by the shear stress, and supports that continuous exchange of BSM molecules between bulk solution and on the substrate is a characteristic tribological behavior of the macroscale contact in BSM solution.

3.4 Nanoscale lubricating properties of BSM

Figure 5a presents the friction vs load plots obtained from the BSM samples, ae-BSM/BSA mixture, and BSA by FFM. Friction forces showed fairly linear increase with increasing load with
nearly zero friction at zero applied load for all cases. Thus, the coefficients of friction for these systems, $\mu$, were obtained from the slopes in friction-vs-load plots. The results are shown in Table 1. As can be seen in Figure 5a, the nanoscale frictional properties of the BSM solutions follow the same trend with those on the macroscale, with friction increasing in the order $\text{ae-BSM} < \text{d-BSM} < \text{ar-BSM} < \text{ae-BSM/BSA} < \text{BSA}$. Figure 5b shows the dependence of $\mu$ on the tip scanning velocity ($v$) for $\text{ae-BSM}$, $\text{ar-BSM}$ and $\text{BSA}$. For all cases, $\mu$ shows a viscous frictional behavior, i.e., increasing with increasing speed. In particular, the slopes of linear fits in the graphs (d$\mu$/dv, shown in Table 1) indicate that the viscosity of the adsorbed layer increases in the order of $\text{ae-BSM} < \text{ar-BSM} < \text{BSA}$. For the tip to plow through the adsorbed chains, the bonds between BSMs and those of BSM-substrate have to be disrupted. A more hydrated layer, e.g. $\text{ae-BSM}$, possesses higher free volume and conformational flexibility that assist to maintain or reform the bonds with the substrate and with neighboring chains, resulting in a less viscous, quickly recovering network (Figure 5b).

3.5 Macro vs Nanoscale lubrication

Despite the same relative ranking in $\mu$ values among various BSM and BSA solutions, the $\mu$ values of the BSMs and BSA on nanoscale contacts were higher than that in PBS buffer (Figure 5a), which is in sheer contrast to macroscale contact (Figure 4). Apart from different contact area and contact pressure, it is notable that the interfacial hydrophobicity of the tribological interface was different for the two sets of experiments; in the tribometer experiments, the interface was PDMS/PDMS, i.e., hydrophobic/hydrophobic (HB/HB), whilst in FFM experiments, the interface was Si$_3$N$_4$/PDMS, i.e., hydrophilic/hydrophobic (HL/HB). Thus, Si$_3$N$_4$/PDMS tribopair (HL/HB) was employed for tribometer experiments. Conversely, an OTS-modified AFM probe/PDMS was employed for FFM experiments to provide a HB/HB interface on the nanoscale contact. For these experiments, ar-BSM was employed.
3.5.1 Macroscale contact of Si$_3$N$_4$/PDMS (HL/HB) tribopair

As shown in Figure 6, the macroscale contact of Si$_3$N$_4$/PDMS pair revealed lower friction forces than PDMS/PDMS pair in PBS buffer, presumably due to lower adhesive interaction. More importantly, ar-BSM lowered the friction forces not only of the sliding contact of PDMS/PDMS, but also Si$_3$N$_4$/PDMS interface. For example, at 0.25 mm/s, $\mu$ dropped by a factor of ca. 9. This is in clear contrast to the FFM data (Figure 5) where $\mu$ increased by a factor of 1.7 for the same pair in ar-BSM solution.

This scale-dependent lubricating efficacy can be explained by considering the difference in contact area/contact pressure and the number of BSM molecules within the contact area. Assuming Hertzian contact mechanics with Young’s modulus/Poisson ratio of 2.7 MPa/0.5 for PDMS, 8 GPa/0.4 for OTS, and 160 GPa/0.25 for Si$_3$N$_4$ [44], the contact diameter at the macroscale is 0.85 mm for Si$_3$N$_4$/PDMS, and 1 mm for the PDMS/PDMS. At the nanoscale, the contact diameter is estimated to be ca. 80 nm for both Si$_3$N$_4$/PDMS and OTS/PDMS at 30 nN applied load. If we further assume a molecular weight of 1 MDa and spherical configuration for BSM, the diameter of the projected area of BSM on PDMS surface is ca. 65 nm according to the adsorption data (Figure 3). Thus, the number of BSM molecules in the contact area for the tribometer experiments is on the order of $10^4$, whereas it is ca.1.4 only for the FFM experiments. In addition, the contact pressures in tribometer experiments, 0.6 and 1.2 MPa (PDMS/PDMS and Si$_3$N$_4$/PDMS), are lower than those in the FFM experiments, 1 to 4 MPa (corresponding to 1 to 60 nN). Thus, at the macroscale, it is unlikely that all $10^4$ BSM molecules are simultaneously sheared off in a sliding contact with the pin, and a part of the contact area can be still shielded by the adsorbed BSM molecules. In the case of FFM, however, only one or two BSM molecules may be sheared off, or pushed aside, when the tip
ploughs through the BSM films on PDMS slab, which may require more energy than to slide on bare PDMS slab.

**3.5.2 Nanoscale contact of OTS/PDMS (HB/HB) tribopair**

FFM experiments with OTS/PDMS interface have also shown notable differences compared to Si₃N₄/PDMS interface in buffer solution. As shown in Figure 7a, the high adhesion of the OTS/PDMS interface allows the system to remain in contact down to “negative” loads, and also gives rise to a sublinear response of friction to load, which is typical for adhesion-dominated friction regime [47]. However, unlike the case of Si₃N₄/PDMS interface, ar-BSM solution lowered the adhesion and friction of the interface. Analysis of the contact characteristic according to the general transition equation [48], a pull-off force of -16.2 ± 0.2 nN and -7.2 ± 0.5 nN were obtained in buffer and in ar-BSM solution, respectively. However, the transition factor, 0.65 ± 0.06, and the shear strength, 2.8 ± 0.2 MPa, were identical for both solutions. This indicates that the introduction of BSM in the contacts resulted in lowering the adhesion of the system, hence the friction forces too, but the mechanical characteristics of the contact remained unaltered; due to the high contact pressure, the AFM tip can penetrate through the ar-BSM layer and can slide directly on the PDMS surface.

**3.5.3 Solution vs coating at nanoscale contact**

Lastly, regarding the differences in the lubrication mechanisms of BSM at the macro- and nanoscale contacts, it is also important to clarify if a “self-healing” phenomenon is taking place at the nanoscale contact too. Thus, the same experiments shown in section 3.3.2, i.e. solution vs coating experiments, were performed with FFM. The friction-vs-load plots obtained from a layer of ar-BSM coating in PBS buffer and those from ar-BSM solution are presented in Figure 7b. In
contrast to macroscale contacts, the tribological properties of ar-BSM on PDMS surface were measured to be identical in ar-BSM solution and in PBS buffer at both Si₃N₄/PDMS and OTS/PDMS interfaces. As mentioned above, this is mainly because the number of BSM molecules that are under shear stress is limited to one or two because of the extremely small contact area, and thus no significant exchange between BSM molecules on the surface and in bulk solution can occur under tribological contacts.

This observation clarifies that the tribostress-induced “desorption-readsorption” cycle is not present or is of ignorable importance at nanoscale contacts. Thus, varying frictional forces observed from different BSM and BSA layers (Figure 5) at the nanoscale should originate from different physicochemical properties of the layers. Given that AFM probes plough through the BSM layer at the nanoscale contacts, friction forces observed from BSM with different magnitude of “impurities” may reflect the energy dissipation during ploughing. As shown in Figure 5b, friction forces for BSM layers obtained at nanoscale increased with increasing sliding speed. The viscous drag from scanning of an AFM probe within mucin layers adsorbed on the surface is correlated to their diffusive, expanded, and highly hydrated conformation on hydrophobic surfaces [22]. In contrast, BSA has been found to adopt a flattened conformation, resulting in a stiff, high viscosity, compact layer on hydrophobic surfaces [22,45]. As shown in Figure 5b, as the content of BSA increases, the increase of friction with increasing speed (dμ/dv) also increases. Similarly, increasing μ values observed from BSM solution with higher content of impurities, including BSA, (Figure 5a) for the Si₃N₄/PDMS tribopair can be attributed to the increasing energy cost for the tip to plough through adsorbed layers of increasing stiffness.

5. Conclusions
The tribological properties of BSM with varying purification grades, namely ae-BSM, d-BSM, and ar-BSM, were examined at both macro- and nanoscale contacts, and at both HB/HB and HL/HB interfaces. At macroscale contacts, all BSMs effectively lubricated the sliding contacts of both HB/HB and HL/HB interfaces, due to the involvement of a large number of BSM molecules in the contact area. ae-BSM was most effective with \( \mu \) being at the limits of detectability, as low as 0.003, followed by d-BSM and ar-BSM. In macroscale pin-on-disc tribometry experiments, since continuous desorption and re-adsorption cycle of the molecules in the contact area is expected, relatively inferior lubricity of ar-BSM can be explained mainly by the fast adsorption and the dominance of lighter and non-slippery molecules, such as BSA. The lubricating properties of BSM were fairly different at the nanoscale; for the HB/HB interface, BSM did lower the interfacial friction forces, but not as greatly as at the macroscale. In contrast, for the HL/HB interface, the presence of BSMs rather increased the friction forces, and yet, the \( \mu \) values followed the same order from that at the macroscale contacts. Inefficient lubrication of BSM at the nanoscale contact is mainly due to extremely small number of BSM molecules involved in the contact area. Moreover, high contact pressure at the nanoscale contact allowed the AFM probe to penetrate through BSM layer and make a direct contact with underlying substrates for both HB/HB and HL/HB interfaces, and finally required higher energy cost to plough through BSM layers. Higher friction forces observed from BSM containing larger amount of BSA at the nanoscale cannot be explained by the faster adsorption of lighter molecules at the contacting area, since no significant desorption from tribostress as observed in macroscale contacts is expected in the first place. Instead, higher increasing rates of coefficient of friction with increasing speed, \( d\mu/dv \) (Table 1), for BSA and BSA-rich BSMs suggests that their higher stiffness and viscous drag are responsible for higher friction compared to BSM layer comprised of purified BSM only, i.e. ae-BSM.
Acknowledgement

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References

FIGURE CAPTIONS

**Fig. 1** Coomassie-stained SDS-PAGE of ar-BSM, d-BSM, and ae-BSM. The concentration of the loaded samples was 2 mg/mL.

**Fig. 2** Hydrodynamic diameter distribution of BSM samples as measured by DLS: ar-BSM, d-BSM, ae-BSM, ae-BSM/BSA (20:1 v:v).

**Fig. 3** Surface adsorption profiles of ar-BSM, d-BSM, ae-BSM onto PDMS surface obtained by OWLS.

**Fig. 4** $\mu$-vs-speed plots for the PDMS/PDMS tribopair in PBS buffer and BSM solutions as measured by pin-on-disc tribometry.

**Fig. 5** (a) Friction-vs-load plots obtained from Si$_3$N$_4$ probe/PDMS slab contacts in PBS buffer and BSM solutions (1 mg/mL), (b) $\mu$-vs-speed plots obtained from Si$_3$N$_4$ probe/PDMS contacts in PBS buffer and BSM solutions (1 mg/mL) obtained by FFM.

**Fig. 6** $\mu$-vs-speed plots for the sliding contacts of PDMS/PDMS and Si$_3$N$_4$/PDMS contacts in PBS buffer and ar-BSM solution (1 mg/mL) as measured with pin-on-disc tribometry.

**Fig. 7** (a) Friction-vs-load plots obtained from OTS/PDMS and Si$_3$N$_4$/PDMS contacts in PBS buffer and ar-BSM solution (1 mg/mL) as characterized with FFM (b) Friction-vs-load plots obtained from a layer of ar-BSM coating on PDMS slab in PBS buffer and in ar-BSM solution (1
mg/mL) as characterized with FFM. Both OTS-modified Si$_3$N$_4$ probe and Si$_3$N$_4$ probe were employed.
Fig1.
Fig 2.
Fig 3.
Fig 4.

![Graph showing coefficient of friction vs speed (mm/s)].

- PBS: Open circles
- BSA: Green circles
- ae-BSM/BSA: Green squares
- ar-BSM: Black circles
- d-BSM: Red circles
- ae-BSM: Blue circles
Fig 5.
Fig 6.
Fig 7.
Table 1. Coefficient of friction ($\mu$) obtained from linear fits to the frictions-vs-load plots (Figure 5a) and its dependence on speed ($d\mu/dv$) obtained from $\mu$-vs-speed ($v$) plots (Figure 5b) as characterized with FFM.

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Interactions between bovine serum albumin and bovine submaxillary mucin in solution: A spectroscopic study

SDS-PAGE and MALDI-TOF MS analysis identified bovine serum albumin as being the most abundant non-mucin protein present in commercial. Furthermore, the content of BSA in commercial BSM has previously been determined to be 9% by weight. In this study, we were interested in elucidating the mode of interaction between BSM and BSA. The results were interesting albeit somewhat inconclusive. Further experimentation has therefore been planned in the form of nuclear magnetic resonance studies and changes in the ratio of the proteins.

My contributions to this study were purification of BSM, fluorescence spectroscopy measurements, dynamic light scattering measurements and authoring the manuscript.
Interactions between bovine serum albumin and bovine submaxillary mucin in solution: A spectroscopic study

Jan Busk Madsen,¹ Kirsi I. Pakkanen,¹ and Seunghwan Lee¹

¹Department of Mechanical Engineering, Section for Materials and Surface Engineering, Technical University of Denmark

KEYWORDS: bovine submaxillary mucin (BSM), bovine serum albumin (BSA), Protein interaction
Abstract
The in-solution interactions between bovine submaxillary mucin (BSM) and bovine serum albumin (BSA) were investigated using spectroscopic techniques. Tryptophan fluorescence from BSA was monitored by fluorescence (FL) spectroscopy while dynamic light scattering (DLS) was employed to determine changes in the hydrodynamic size of the molecules. When mixing the proteins immediately, the FL spectrum is dominated by the fluorescence signal from BSA. After one week of incubation at 4 °C, a drop in signal intensity and a red shift towards higher wavelengths was observed, indicating that the BSM BSA interaction may shield the BSA tryptophan from solution. DLS showed a slight shift in size in the main BSM peak indicating incorporation of BSA. BSM was incubated for 21 days prior to adding BSA. After adding BSA and further incubation, a blue shift towards lower wavelengths and a 50% signal intensity drop occurred. This indicates an increase in solvent exposure for the BSA tryptophan and may be an indication of a different mode of interaction. DLS showed a reduction in the spread of the hydrodynamic sizes.

Introduction
As the major component, mucins make up the bulk of the mucus matrix. Mucus gels are found throughout the body where they among other functions protect the underlining epithelial layers from physical abrasion by providing aqueous lubrication. Furthermore, it acts as physical barrier towards microorganisms thus contributing to diminishing occurrences of infection. The tertiary structure of mucins is generally described as having a “bottlebrush” like conformation where globular C- and N- terminals are separated by heavily glycosylated central domains. In the terminal domains several structural motifs have been identified in the primary sequence such as Von Willebrand like domains, cysD domains and cysteine knots. The central glycosylated regions consist mostly of tripeptide repeats of proline, threonine and serine known as PTS domains. In the PTS domains, either the side chain of threonine or serine carries O-linked mucin type
glycosylations. The majority of the carbohydrate moieties attached to the central domain consist of oligosaccharides containing 2-3 carbohydrates; however, larger branched oligosaccharides have also been identified to be intermingled in the structure. Of the complete mucin macromolecule, the carbohydrate moieties make up 50-80% of the total molecular weight which also facilitates the lubrication properties of the mucins and mucus gels. Hydrogen bond interactions between the central domain carbohydrate structures and water in the environment enable formation of aqueous lubricating films thus lowering interfacial friction forces. Mucins have also displayed other interesting surface and interfacial functions as part of drug delivery systems and antibacterial coatings. Other biomolecules such as lipids, proteases, amylases and antigens are known to interact with and incorporate into the mucus lining. Here, the proteins further facilitate the function of the mucus lining as an antibacterial coating by combating migration of bacteria across the mucus barrier. Ex situ studies using purified mucins have indicated that the gel formation capabilities of purified mucins have been eliminated at some point during the purification process. The presence of other biomolecules may therefore play a significant role in the gel formation. BSA has been reported to be an impurity that is present commercial BSM. Whether BSA is an integral part of the mucus matrix or the BSA content in commercial BSM is an artifact of purification has yet to be determined. A previous study using quartz crystal microbalance dissipation found that a pre-adsorbed layer of BSA prevented subsequent BSM adsorption. However, if BSM was allowed to adsorb onto the surface first, subsequent addition of BSA resulted in complex formation while a mixture of the protein showed intermediate layer formation. Rheological studies of mucin and BSA in solution also showed that a strong interaction between the proteins occurred. Moreover, commercially available mucins, in particular BSM, have shown that an un-ignorable amount of BSA is integrated into the sample (roughly 9 % of total sample by weight), which is not easily removed by simple filtration or dialysis. To date, no
spectrophotometric methods have been applied to further elucidate the interaction between BSM and BSA. Due to BSM and BSA both displaying polyanionic properties at neutral pH, no electrostatic interactions between the proteins are expected to occur in solution. However, both proteins are also known to have hydrophobic patches present on their surfaces and non-specific protein-protein interactions may therefore take place. Using fluorescence spectroscopy (FL) and dynamic light scattering (DLS), we were interested in elucidating the manner and rapidity of the interactions occurring between BSM and BSA. To this end, it was prudent to ensure the highest obtainable degree of purity of the BSM. Using a recently developed purification protocol, Commercial BSM was isolated further ensuring that any interactions observed would be BSM-BSA specific. Experiments were performed at a ratio of 10:1 between BSM and BSA which corresponds to that of commercial BSM.

Materials and methods

Bovine submaxillary mucin (BSM) and bovine serum albumin (BSA)

BSM (M3895-1G, type I-S, lot 039K7003V) and BSA were purchased from Sigma Aldrich (St. Louis, MO). The concentration of the BSM samples used in this study was 1 mg/mL, while BSM and BSA were mixed at a ratio of 10:1 (1 mg/mL: 0.1 mg/mL).

Purification of BSM

BSM was purified as described in a previous study. BSM was dissolved overnight at 4 °C on a nutating mixer in a Na-acetate buffer (10 mM Na-acetate, 1 mM EDTA, pH 5.0) to a final concentration of 10 mg/mL. The solution was clarified by sterile filtration (5 µm hydrophilic polyethersulfone sterile filter; Pall Corporation, United Kingdom.) followed fractionation according to their charge on a high load 16/26 Q Sepharose high performance anion exchange column (GE Healthcare Life Sciences, Sweden) installed on an Äkta Avant Chromatograph (GE Healthcare,
Fractionation of bound proteins was achieved with a multi-step gradient of high salt Na-acetate elution buffer (10 mM Na-acetate, 1 mM EDTA, 1.2 M NaCl, pH 5.0). Fractionated proteins were analyzed by SDS-PAGE and stained using coomassie brilliant blue to visualize proteins or periodic acid/Schiff staining to identify fractions with glycoproteins. BSM fractions were pooled, dialyzed against milliQ grade water and subsequently freeze-dried. All steps were performed at 4 °C to minimize potential protelytic degradation.

Fluorescence spectroscopy (FL)

Intrinsic tryptophan fluorescence of BSA in the absence and presence of BSM was measured using the fluorescence set-up of the Chirascan spectrophotometer (Applied Photophysics, UK). Excitation wavelength, 280 nm, was selected to minimize tyrosine contribution to fluorescence to allow more accurate analysis of tryptophan fluorescence. Voltage of the light source was kept constant for all samples. Emission was collected between 320 and 380 nm with a 1 nm step and 1 nm bandwidth. All spectra measured were average of three scans. The samples were measured in a rectangular quartz cuvette with 10 mm path length (Hellma, Germany).

Dynamic light scattering (DLS)

Samples were prepared in 0.22 µm-filtered buffers and measured using a Malvern Zetasizer Nano ZS two angle particle and molecular size analyzer (Malvern Instruments, United Kingdom). Non-invasive back scatter (NIBS, measurement angle 173°) was used to achieve the highest sensitivity. A He-Ne laser at 633 nm was used as a light source and the temperature was set at 25 °C. Disposable cuvettes (PMMA, Plastibrand) were employed for DLS measurements. Each sample was measured in triplicates. The Malvern Zetasizer software (Version 7.02) was used to analyze the obtained data and DLS data was plotted according to the intensity distribution of the hydrodynamic diameter (Dh).
Results and discussion

Stability of BSM

Circular dichroism (CD) spectroscopy was employed to investigate the long-term stability of BSM (data not shown) at 4 °C. Common for all spectra were a small positive maximum at 220 nm and a stronger negative minimum at 203 nm with comparable intensity. CD spectra of BSM with similar secondary structural features have previously been reported. The far-UV CD spectrum of BSM was recorded every 7 days for a total of 21 days. No noticeable change in the spectra was observed over the full 21 days, indicating that no protein degradation had occurred during the incubation. A strong stability of BSM against heating was also confirmed in a previous study. General structural stability of BSM, compared to globular proteins, may be ascribed to lack of higher-order secondary structure in BSM.

Interactions between BSM and BSA in solution

Fluorescence spectroscopy on proteins focuses mainly on the intrinsic emission from the aromatic side chains of tyrosine and tryptophan (Trp) and to a lesser extent phenylalanine. However, as Trp emission has a higher intensity, and is much more sensitive to changes in its microenvironment, its emission usually dominates the spectrum. Primary and tertiary structure determination has established that BSA contains two Trp residues, Trp-134 and Trp-213, which makes it suitable for tryptophan fluorescence analysis. One of the Trp residues is located at the bottom of a hydrophobic cleft whereas the other is positioned on the surface on the molecule and therefore accessible to the aqueous solvent. Fluorescence spectroscopy was then used to monitor fluorescence emission from the mixture of BSM and BSA. From the fluorescent emission shift from Trp residues, it is possible to monitor whether interaction between BSA and BSM is occurring. BSM and BSA was mixed at a ratio of 10:1 (as commercial BSM has previously been reported to contain up to 9% BSA by weight), and subsequent measurements showed a change in the...
fluorescence emission signals (Black solid emission spectrum, Figure 1A) compared to BSM only (Figure 1, blue emission spectrum), indicating that free BSA is present in the BSM-BSA mixture sample. The spectrum is comparable to that of BSA alone (Figure S1 in the supplementary information). After incubation at 4 °C for 7 days, a substantial decrease by approximately 25% of the fluorescence emission intensity was observed (Block dotted emission spectrum, Figure 1A). This was not observed for BSA alone, where BSA displayed a slightly increased intensity in the emission spectrum and no shift in the peak position (Figure S1). The drop in emission intensity along with a slight red shift in the peak maximum (341 nm to 344 nm) signify that a conformational change in the BSA rendered the surface Trp residue to be more exposed to the water. Although a shift in the wavelength region close to 350 nm may also be due to denaturation, it is most likely not the case here as CD spectroscopy showed that no denaturation of BSM, the major component, occurs within the timeframe. The interaction was further verified by DLS. Figure 1B shows the peak intensity plots for the BSM-BSA mixture. In the freshly mixed sample a shoulder peak at 8 nm corresponding to BSA was present next to the main BSM peak (z-average of ~69 nm). After 7 days, the BSA peak intensity was slightly reduced. The size reduction in the BSM main peak could be an indication of BSM-BSA complex formation and that the complex formation could have impacted the “loose” and non-structured configuration of the BSM. Porcine submaxillary mucin forms inter-molecular disulfide linkages through the terminal domain regions. Considering that the BSM and BSA were mixed immediately prior to the first measurement, BSA may potentially interact with the initially non-linked terminal peptide domains of BSM, rather than the glycosylated central domains, as BSM-BSM terminal domain interactions formations may be time dependent.
Figure 1 A) Fluorescence emission spectrum of BSM and BSA freshly mixed at a ratio of 10:1 (Black solid line) and freshly dissolved BSM (blue). The dashed black line shows the emission spectrum after incubation at 4 °C for 7 days. B) DLS measurements of the hydrodynamic size. Samples as in A.

The lack of gel-forming properties of commercial BSM has previously been reported. BSM was incubated at 4 °C for 21 days to examine if any changes, such as self-assembly and formation of larger aggregates, occurred to the molecules while in solution. The DLS measurements shown in Figure 2B verified that no aggregation had occurred between BSMs incubated in solution for 21 days. Furthermore, it showed identical maximum peak intensity as that of the freshly dissolved sample. The Trp fluorescence of BSM only was also measured. Both the freshly dissolved BSM and the “aged” BSM showed low fluorescence signals with identical emission maxima (Figure 2A). After 21 days, the intensity of fluorescence emission was lowered by ~20% and may indicate a
change in the molecular conformation of BSM. A partial sequencing of 1323 amino acid residues
from BSM was found to contain 6 Trp residues.\textsuperscript{38} A combination of the scarcity of Trp residues in
the protein combined with extended incubation in solution may explain the emission quenching.
Even though DLS showed that the hydrodynamic size of the BSM did not increase, the prolonged
hydration of the molecules may induce an overall change in the tertiary structural conformation,
leading to further burial of the Trp residues in the three dimensional configuration of BSM.

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{FL (A) and DLS (B) measurements of BSM freshly dissolved (solid lines) and after 21 days of incubation at 4 °C (dashed lines).}
\end{figure}

The data presented in Figure 3 shows the interactions between “aged” BSM that was incubated in
solution with BSA at 4 °C for 21 days. Fluorescence spectroscopy revealed a significant drop in the
fluorescence emission of ~50% after being incubated with the “aged” BSM for 7 days. This
indicates that an interaction between the proteins took place. Furthermore, the emission maximum had a blue shifted emission maximum from 345 nm to 342 nm. The shift towards a lower emission wavelength suggests the Trp residues in BSA are being shielded from surrounding solvent. This is in contrast to the freshly mixed BSM-BSA sample where a red emission shift was observed (Figure 1A). The emission shift towards the blue wavelength and thereby Trp residues being more shielded may indicate that the “naked” terminal peptide domains in the BSM may in fact be interacting with other BSM, shifting the majority of BSM-BSA interactions to possibly occur between BSA and the oligosaccharides of the central domain. Given enough time, the BSM conformation may have changed due to either BSM-BSM interactions or full hydration of the oligosaccharide moieties even though DLS measurements showed no significant increase in the hydrodynamic size of the molecular species in solution (Figure 2B and Figure 3B). Interactions within the glycosylated region of BSM could explain the shielding observed as the surface Trp of the BSA could be “buried” between the oligosaccharide chains. The emission shift between the two samples could indicate that a protein-protein interaction is preferable rather than protein-carbohydrate. After being incubated for a week with BSA, DLS measurements of the “aged” BSM showed an intensity increase and move towards a more uniform hydrodynamic size (Figure 3B), even though the average $D_h$ did not change. The peaks for the molecular species smaller than 15 nm and larger than 80 nm became weaker in comparison to the measurement immediately after addition of BSA (Figure 3B). The reduction of the lower hydrodynamic sizes is most likely due to complex formation of BSA into the BSM. It may well be that the quaternary structural interactions between BSM and BSA decreases the rotational degree of freedom of the unstructured BSM central domains thus “stiffening” the tertiary structure of BSM and limiting its hydrodynamic range.
Figure 3 FL (A) and DLS (B) measurements of “aged” BSM with added BSA (solid lines) and after 7 days incubation at 4 °C (dashed lines)

Conclusion

Commercially available BSM is known to contain other biomolecules. A mild purification protocol was employed to select for uniform molecular weight fraction of BSM and extensively reducing the presence of other biomolecules. BSA has previously been reported to interact with mucins and may be an integrated part of the mucus gel matrix. A previous study focused on the surface rheological behavior of BSM in association with BSA. Here we investigated the interaction between BSM and BSA in solution using spectroscopic approaches. When freshly mixed, a complex between the peptide terminal domains of BSM and BSA may have formed. Fluorescence from BSA showed a red shift towards a higher wavelength maximum in the emission spectrum indicating more solvent accessible Trp residues and thus an alteration of the protein
conformation. DLS revealed a reduction in the BSA secondary peak and a slight reduction in the z-average of the hydrodynamic size of the BSM main peak which may be indicative of complex formation between BSM and BSA. BSM was “aged” by incubation for 21 days at 4 °C. “Ageing” did not impact the stability of the BSM and also did not lead to aggregation as no increase in hydrodynamic size was observed. Addition of BSA to the “aged” BSM increased the fluorescence emission Spectrum compared to “aged” BSM alone. However, after 7 days of incubation at 4 °C, the emission spectrum intensity decreased by ca. 50%, thus suggesting an interaction between the proteins may have occurred. Furthermore, a blue shift to a lower wavelength occurred, indicating that the Trp residues present in BSA became more shielded from water. This may be due to protein-oligosaccharide interactions between BSA and the glycosylated central domain of BSM. The decrease observed could originate from BSA being “buried” in the carbohydrate side chains of BSM and the charge effects of the oligosaccharide chains could contribute to quenching of the signal. DLS showed that complex formation between “aged” BSM and BSA may potentially affect the rotational freedom of BSM as a slight reduction in the spread of the higher hydrodynamic sizes recorded were reduced.

In conclusion, no extensive complex formation between BSM molecules appears to be occurring as recorded by DLS. However, a time-dependent interaction or rearrangement of the BSM molecules does occur as the interaction with BSA changes depending on the time in solution. The data presented here may indicate that BSM-BSA interactions are non-specific. However, the data may also indicate that BSA preferentially interacts with the non-glycosylated domains of BSM rather than the heavily glycosylated central domain as suggested by fluorescence spectroscopy.
References


Supplementary information

Figure S1 Fluorescence emission Spectra of freshly mixed BSA (solid) and BSA aged for one week (dashed)
Thermostability of bovine submaxillary mucin (BSM) in bulk solution and at a sliding interface

It is commonly accepted that most protein denature above a certain threshold temperature. Upon denaturation they usually also lose their biological function. In this study, we were interested in monitoring the changes in the structure of BSM by heating and cooling, both in situ and ex situ, and how it affected adsorption and tribological properties of the BSM.

My contributions to the study was the preparation of BSM in the form of dialysis, adsorption measurements by OWLS, ex situ CD spectroscopy measurements, PoD- and MTM tribological measurements and authoring the paper.
Thermostability of bovine submaxillary mucin (BSM) in bulk solution and at a sliding interface

Jan Busk Madsen, Kirs I. Pakkanen, Seunghwan Lee*

Department of Mechanical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

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A B S T R A C T
Thermostability of bovine submaxillary mucin (BSM) was studied in terms of its structure, hydrodynamic size, surface adsorption, and lubricating properties in the temperature range of 5–85 °C. The overall random coil structure of BSM showed a gradual loosening with increasing temperature as characterized by circular dichroism (CD) spectroscopy, but this change was fully reversible upon lowering temperature. Extended heating up to 120 min at 80 °C did not make any appreciable changes in the structure of BSM when it was cooled to room temperature. The hydrodynamic size of BSM, as studied by dynamic light scattering (DLS), showed a slight increase after heating at high temperature (80 °C). Optical waveguide lightmode spectroscopy (OWLS) studies showed facile adsorption of BSM onto poly(dimethylsiloxane) (PDMS) surface (>180 ng/cm²) at room temperature due to its amphiphilic characteristics. Adsorbed mass of BSM was noticeably reduced after heating at 80 °C, possibly resulting from its aggregation. BSM showed excellent lubricity at self-mated sliding contacts between PDMS at room temperature or lower (friction coefficient = 0.02), even when BSM solution was pre-heated up to 120 min at 80 °C. Gradual degradation of lubricity of BSM was observed with increasing temperature, but it was also reversibly recovered with decreasing temperature. Structural and functional stability of BSM against heating is proposed to originate from heavy glycosylation and lack of higher degree of protein structure in BSM.

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1. Introduction

Many proteins are known to be thermolabile causing them to partially unfold, and in many cases to form aggregates, when exposed to temperatures higher (and sometimes lower) than their physiological temperature range [1–5]. Protein unfolding is metabolically expensive for an organism and therefore a highly undesirable occurrence. Spontaneous refolding of the polypeptide chain upon return to physiological conditions often leads to a misfolded protein and the subsequent loss of protein function [5].

Mucous gels are found on various epithelial surfaces throughout the body, such as the intestines, lungs and oculars [6–9]. The major component comprising mucous gels are mucins. Mucins are high molecular weight macromolecules in the MDa molecular weight range consisting of an apomucin peptide chain heavily modified with post-translational glycosylations that make up between 50% and 80% of its total molecular weight [10–12]. Structurally, mucins include large heavily glycosylated central domains and minor unglycosylated, globular terminal domains, thus displaying an overall "bottlebrush"-like conformation [11]. The central domains are sequence rich in proline, threonine, and serine, and are known as mucin-type PTS domains. Either the threonine or serine amino acid residue in the PTS motif is linked with glycans via mucin type O-glycosylation [13]. The glycosidic modifications facilitate the recruitment of water by mucins to form viscous mucus gels that provide a protective barrier against pathogens and mechanical insult [7–9]. This also generates a slippery interface between the mucous-covered tissues or between mucous-covered tissue and other objects. In addition to its biological functions, the bioengineering potential of mucins has recently started to attract attention, for instance as a source of biocompatible surface coatings that suppress bacterial adhesion or that induce favorable immune cell responses [14–18]. Due to their unique lubricity, mucins are included as a key component of saliva substitutes [19,20]. The majority of mucin coating studies for biomaterials has been carried out on hydrophobic, polymeric surfaces [14–18,21–23]. The amphiphilic characteristics of mucins are known to allow for spontaneous adsorption onto hydrophobic surfaces from aqueous solution [22–25]; mucin interacts with surfaces through the hydrophobic N- and C-termini while the heavily glycosylated domains in the central region of the polypeptide interact with the aqueous environment [13,21–23,25,26].

In this study, we have investigated the thermostability of bovine submaxillary mucin (BSM) for its potential application as...
a coating for polymeric biomaterials. While the influence of heating on the structural stability of BSM was firstly investigated, the impact on the surface adsorption and lubricating properties of BSM on polymeric biomaterials has also been studied. As mentioned above, most proteins undergo irreversible partial denaturation upon exposure to temperature variations, diverting drastically from their physiological conditions [5]. Generally, the vast majority of studies on the thermostability of proteins has been performed on globular proteins [1–4,27], although some smaller glycoproteins have been shown to be highly heat stable [28,29]. Large glycoproteins, such as mucins, on the other hand, diverge significantly in their structural conformation from globular proteins and elucidation of their structural stability at high temperatures has been studied to a lesser degree [30]. The overall secondary structure of BSM is known to consist mainly of random coil structural motifs while no tertiary structural interactions have been observed. This is not surprising as the vast majority of BSM is made up of the denatured coiled, leaving only the terminal domains to possibly display a globular conformation. Thus, any higher level of structural complexity is not readily expected from BSM [13]. It is of central interest in this study to elucidate how these secondary structural features impact the structural and functional stability of mucins during and after heat treatment.

2. Materials and methods

2.1. Bovine submaxillary mucin (BSM) and bovine serum albumin (BSA)

BSM (M3895-1G, type I-S, lot 039K7003V) and BSA were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Dialysis and thermal treatments of BSM

BSM was dissolved in MilliQ grade water and dialysed in a Float-a-lyzer dialysis tube (cut-off 100 kDa) against MilliQ grade water to reduce the concentration of non-mucin protein content. “Impurities” present in the BSM as received from the manufacturer were partially removed, but a noticeable amount of them, including serum albumin (BSA), was detected by Coomassie-stained SDS–PAGE [31]. BSM was subsequently lyophilized and dissolved in phosphate buffered saline (PBS, 10 mM, 137 mM NaCl, pH 7.4) to a final concentration of 1 mg/mL. Thermal treatments were carried out in two ways; firstly, the temperature of BSM solution was ramped from low temperature (5 °C) to high temperature (85 °C), followed by subsequent cooling back to low temperature (5 °C). The structural and lubricating properties of BSM were investigated by means of circular dichroism (CD) spectroscopy and mini-traction machine (MTM), respectively, at 5 or 10 °C intervals. Further details are addressed in the relevant sections below. Secondly, the BSM solutions were exposed to high temperatures for varied durations; vials containing BSM solutions were submerged in a water bath either at 37 °C for 10 min or at 80 °C for 10 min, 30 min or 120 min, respectively, and subsequently cooled to room temperature. The structural, surface adsorption, and lubricating properties of heat-treated BSM were characterized by means of CD spectroscopy/dynamic light scattering (DLS), optical waveguide lightmode spectroscopy (OWLS), and pin-on-disc tribometry, respectively, at room temperature.

2.3. Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectra of BSM solution were acquired employing a rectangular quartz cuvette with 0.5 mm path length (Hellma GmbH & Co. KG, Müllheim, Germany) using Chirascan spectrophotometer (Applied Photophysics Ltd., Surrey, UK). Concentration of BSM in the cuvette was 1 mg/mL in PBS. Temperature was set using a CS/PCS single cell Peltier temperature controller (Applied Photophysics Ltd., Surrey, UK) and the temperature scan was programmed using the Chirascan software. For the measurements at varying temperatures, temperature was set to 5 °C in the beginning of the measurements and the sample was allowed to equilibrate in this temperature for 10 min. After this, the temperature was increased up to 85 °C at a ramp rate of 2 °C/min and then decreased down to 5 °C. Spectra were recorded at 5 °C temperature intervals. The spectra were recorded in the far-UV region, from 240 to 195 nm with a step size of 2 nm, bandwidth of 1 nm and time-per-point value of 1.5 s. For the measurements at room temperature after heating, the samples were heat-treated as described above and cooled down to room temperature. Spectra of the samples were recorded in the far-UV range from 260 to 195 nm with a step size of 1 nm, a bandwidth of 0.5 nm and a time-per-point value of 1.5 s. One measurement was obtained from averaging three traces. All samples were measured twice and averaged again, and the background from buffer was subtracted.

2.4. Dynamic light scattering (DLS)

BSM samples (1 mg/mL) were prepared in 0.22 µm-filtered PBS buffer, thermally treated as described (room temperature measurements after heating only), and measured using a Malvern Zetasizer Nano ZS two angle particle and molecular size analyzer (Malvern Instruments, Worcestershire, UK). Non-invasive backscatter (NIBS, measurement angle 173°) was used to give the highest sensitivity. The light source is a He–Ne laser at 633 nm and the temperature was set at 25 °C. Disposable cuvettes (PMMA, Plastic-brand) were employed for DLS measurements. Each sample was measured in triplicates. The Malvern Zetasizer software (Version 7.02) was used to analyze the obtained data. All DLS data were illustrated in the form of the intensity distribution of the hydrodynamic diameter (Dh).

2.5. Optical waveguide lightmode spectroscopy (OWLS)

OWLS is based on grating-assisted in-coupling of a plane-polarized He–Ne laser light (633 nm) into a planar waveguide coating (200-nm thick SiO2,5TiO2,76O2 waveguiding layer on 1 mm thick AF 45 glass (Microvacuum Ltd., Budapest, Hungary). Adsorption of biomolecules from bulk liquid to the interfacing solid surface is measured by monitoring the changes in the refractive index at the vicinity of the solid–liquid interface. This method is highly sensitive to a distance of ~200 nm from the surface of the waveguide. The instrument also allows for is situ monitoring of adsorption kinetics as the measurement-time to resolution is only 3 s. OWLS experiments were carried out using the OWLS 210 Label-free Biosensor system (Microvacuum Ltd., Budapest, Hungary).

In order to keep the substrates for surface adsorption studies by OWLS and all the tribology studies (see below) consistent, waveguides used in this work were coated with a layer of poly(dimethylsiloxane) (PDMS). To this end, the waveguides were spin-coated at 2500 rpm for 15 s firstly with an ultrathin layer (ca. 24.3 ± 3.1 nm, determined by scratch test using atomic force microscopy in tapping mode) of poly(dimethylsiloxane) (Sigma Aldrich, St. Louis, MO, USA), dissolved in HPLC grade toluene at 6 mg/mL. This was followed by coating with a subsequent ultrathin layer of PDMS (ca. 16.4 ± 0.17 nm) [32]. The base and curing agent of a commercial silicone elastomer (Sylgard 184 elastomer kit, Dow Corning, Midland, MI, USA) were dissolved in hexane at a ratio of 10:3 (final concentration, 0.5% w/w). The solution was spin-coated onto a waveguide at 2000 rpm for 25 s, and cured in an oven at 70 °C overnight.
The PDMS-coated waveguide was exposed to PBS buffer prior to sample injection until a stable baseline was obtained. A programmable syringe pump (Model 1000-NE, New Era Pump Systems, Inc., NY) was used to pump buffer solutions through a flow-cell over the OWLS waveguide surface. 100 μL of thermally treated BSM sample (1 mg/mL in PBS) was then injected via a loading loop. Upon observing surface adsorption, the pump was stopped so that the BSM molecules could adsorb onto surface under static conditions. After 10 min, the flow cell was rinsed with PBS buffer by restarting pumping. The adsorbed mass densities were calculated according to de Feijter’s equation [33]. The experiment was repeated two or three times for each type of heat-treated BSM. A refractive index increment (dn/dc) value of 0.150 cm²/g for BSM was used for the calculation of the adsorption masses [34].

2.6. Mini-traction machine (MTM)

A mini-traction machine (MTM, PCS Instruments, London, United Kingdom) was employed to characterize the changes in the lubricating properties of BSM (1 mg/mL in PBS) during a 10 °C stepwise temperature increase and decrease ranging between 5 °C and 85 °C. The experiment was performed under the condition of a 2 N load, a speed of 5 mm/s, and for 10 min at every temperature interval. While MTM can be used for mixed rolling/sliding contacts [35], a pure sliding contact was chosen in this study to provide the identical contact characteristics with pin-on-disc tribometry, which was used for room temperature lubricating properties measurements of BSM after heat treatments (see below). A PDMS tribopair was used in this study and was prepared by cast molding of thoroughly mixed base fluid and crosslinker of a Sylgard 184 elastomer kit (Dow Corning, Midland, MI, USA) at a ratio of 10:1. Gentle vacuum was applied to remove any air bubbles generated during mixing. The disc was prepared by casting the PDMS mixture mentioned above into a home-machined aluminum plate as described above. The discs were prepared by casting the PDMS-coated waveguide into a petri dish (Ø = 50 mm). A 96 micro-cast-coating a standard MTM steel disc with an approximately 2.5 mm thick layer of PDMS in a petri dish (Ø = 50 mm). Gentle vacuum was applied to remove crosslinker of a Sylgard 184 elastomer kit (Dow Corning, Midland, MI, USA) at a ratio of 10:1. The PDMS-coated waveguide was exposed to PBS buffer prior to sample injection until a stable baseline was obtained. A programmable syringe pump (Model 1000-NE, New Era Pump Systems, Inc., NY) was used to pump buffer solutions through a flow-cell over the OWLS waveguide surface. 100 μL of thermally treated BSM sample (1 mg/mL in PBS) was then injected via a loading loop. Upon observing surface adsorption, the pump was stopped so that the BSM molecules could adsorb onto surface under static conditions. After 10 min, the flow cell was rinsed with PBS buffer by restarting pumping. The adsorbed mass densities were calculated according to de Feijter’s equation [33]. The experiment was repeated two or three times for each type of heat-treated BSM. A refractive index increment (dn/dc) value of 0.150 cm²/g for BSM was used for the calculation of the adsorption masses [34].

2.7. Pin-on-disc (PoD) tribometry

The influence of heat treatment on the lubricating properties of BSM (1 mg/mL in PBS) was also investigated using a pin-on-disc tribometer (CSM, Peseux, Switzerland) for post-heating measurements. This approach is based on a loaded pin forming a sliding contact with a disc. The load on the pin was controlled by the application of deadweight (5 N). The friction forces between them were measured at a controlled rotation speed of the disc at 2.5 mm/s. Disc rotation was enabled by a motor beneath the disc while the pin remained stationary. Friction generated during sliding contacts was monitored by a strain gauge. Friction forces data were acquired over 100 rotations at a fixed radius of 5 mm. A PDMS tribopair was used for the experiments. The pins were cast as described above. The discs were prepared by casting the PDMS mixture mentioned above into a home-machined aluminum plate with flat wells designed to the dimensions (30 mm diameter × 5 mm thickness) of the tribometer.

3. Results and discussion

3.1. Influence of heat treatment on the secondary structure of BSM and BSA

CD spectroscopy measurements were performed to investigate the influence of heating and subsequent cooling in the temperature range from 5 °C to 85 °C on the secondary structure of BSM. The far-UV spectra (Fig. 1A) of BSM at 5 °C presented a weak positive band at ca. 220 nm and a strong negative band at ca. 200 nm, indicating that the BSM molecules are mostly in a random coil conformation.

This is easily understood in terms of the heavy glycosylation of the BSM polypeptide backbone and consequent difficulty of forming higher order structure [13,26]. As temperature was gradually increased, the negative band at ca. 200 nm started to become weaker in intensity, accompanied by shifting its local minima towards larger wavelengths. In parallel, the positive band at 220 nm also started to lose its intensity and display more and more negative ellipticity at this wavelength. These changes can be indicative of loosening of the random coil structure of BSM as a result of increasing temperature. The structural changes occurred to BSM molecules by heating were, however, not irreversible; BSM started to regain the original structure prior to heat treatment as the temperature was lowered again from the highest value (85 °C). For example, the CD spectra obtained at 5 °C after heating were almost indistinguishable from the one measured at 5 °C prior to heating as shown in the inset of Fig. 1A. Structural changes of BSM in the course of heating and subsequent cooling are also presented by plotting the CD ellipticities at 200 nm and 220 nm as a function of temperature (Fig. 1B). The transition of BSM from a more coiled structure at low temperatures to a less coiled structure at high temperatures tends to be smooth and without any detectable time lag (Fig. 1B). This can be explained in terms of the absence of a higher level of structural complexity other than random coils in BSM in the first place. As in the native state, heat-treated BSM also reveals a random coil structure, although a gradual loosening of the coil was observed with increasing temperature. Importantly, as the coil structure of proteins is determined by the thermodynamic equilibrium of intramolecular interactions only, no irreversible change is imposed on the structure by change in temperature. The N- and C-terminal regions of mucins have been proposed to be more structured, potentially globular, but as these regions constitute only a minor part of mucin compared to the long heavily glycosylated central domains, it is impossible to detect the structural changes in the terminal domains only [13].

The vast majority of globular proteins are known to be thermo-labile and will partially denature upon extended exposure to high temperatures. Their extensive secondary- and tertiary structural motifs have been found to display irreversible partial unfolding upon heating above physiological conditions and in some cases during prolonged cooling [5]. An extensively studied example of a globular protein is bovine serum albumin (BSA) [27]. BSA has also been reported to be present in commercially available BSM at concentrations of up to 9% [36], and it was not completely removed by dialysis in this study [31]. To be able to visualize the difference in structural stability of a globular protein and BSM upon heating, BSA and BSM were exposed to the same thermal treatments. Both BSA and BSM samples were heated at 37 °C for 10 min or 80 °C for 10, 30, and 120 min, respectively. All samples were subsequently cooled to room temperature prior to analysis and compared to samples that had not been exposed to increased temperatures. The untreated and heated BSA samples at 37 °C for 10 min show identical CD spectra with intense signal local minima at 209 nm and 223 nm, indicating the preservation of α-helical secondary structure even after this treatment (Fig. 2A, black and green, respectively). After heating to 80 °C for only 10 min, however, the thermo-labile nature of BSA is apparent and denaturation is observed as signal intensity starts to diminish (Fig. 3A, blue). Continuous heating at 80 °C for 30 min and 120 min, respectively, causes the secondary structure of BSA to denature further as the CD signal is further diminished (Fig. 2A, orange and red). BSM, on the other hand, shows no sign of denaturation introduced by
thermal exposure (Fig. 2B); all samples were found to present spectra similar to that of untreated BSM. Additionally, this observation also indicates that a trace amount of BSA present in BSM samples after dialysis [31] in this study imposes ignorable influence on the structural stability of BSM against heating. It therefore appears that the overall lack of secondary structure other than random coils observed from BSM is advantageous with regards to its overall structural stability, especially when exposed to heat treatment. However, as the heavily glycosylated central PTS-domains make up the majority of the structure, it is not possible to ascertain whether the globular terminal domains have been altered by the thermal treatments.

3.2. Influence of heat treatment on the hydrodynamic diameter of BSM

The effect of heating on the hydrodynamic diameter of BSM, $D_h$, was investigated using DLS. Fig. 3 shows the $Z$-average of hydrodynamic diameters of BSM with or without heat treatment. The BSM heated at 37°C was found to have a $D_h$ of 233 ± 1 nm, which was comparable to that of untreated BSM sample that showed a diameter of 245 ± 56 nm. Following heat treatment at 80°C for 10 min, the $D_h$ of BSM was observed to increase to 312 ± 25 nm. Similar values were obtained from extended heat treatment for 30 and 120 min ($D_h = 325 ± 25$ nm and $306 ± 10$ nm, respectively), indicating that slight aggregation of the BSMs starts to occur at higher temperature. This observation is in strong contrast to a study on porcine gastric mucin (PGM) [30], where a slight reduction in the hydrated radius was observed upon heating to 60°C; “natively open” characteristics of PGM and gradual loss of water molecules.

![Figure 1](image1.png)

**Fig. 1.** (A) Far-UV CD spectra of BSM measured with 5°C temperature step between 5°C–85°C (solid lines) and 85°C–5°C (dashed lines). Inset shows the far-UV CD spectra obtained at 5°C prior to heating (solid black), at 85°C (red), and at 5°C after heating (dashed black). (B) Structural change of BSM monitored by change in CD signal at two wavelengths, 200 nm (black) and 220 nm (grey) as a function of temperature. The heating and cooling are indicated by a solid and dashed line, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

![Figure 2](image2.png)

**Fig. 2.** Influence of thermal treatment on the secondary structure of BSA (A) and BSM (B). (A) Untreated BSA has been indicated in black, while thermally heat treated BSA is indicated in green (37°C/10 min), blue (80°C/10 min), orange (80°C/30 min) and red (80°C/120 min). (B) Secondary structure of BSM after thermal treatment. Same color legend as in A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

![Figure 3](image3.png)

**Fig. 3.** $Z$-averages measured by DLS for heat treated BSM samples. All values presented are averages of two measurements and error bars indicate standard deviation.
with increasing temperature were provided as an explanation. The contrasting changes in hydrodynamic size of the two mucins against heating can be related to potential difference in glycosylation and also hydration characteristics of the two mucins. Detailed heating conditions may be another reason. More importantly, the PGM molecules reported by McColl et al. are very small in size (24 nm in diameter at 25 °C) [30], and they appear to remain non-associating with each other even after heating. Thus, thermal contraction observed from PGM [30] is reflecting the conformational changes of individual PGM molecules by heating. Meanwhile, the average diameter of BSM in this study is nearly ten-fold larger than that reported for PGM [30], and the size distribution is very broad and ranges from ca. 10 nm to 1000 nm (see Fig. S1 in Supplementary Information). The polydisperse characteristic of BSM at room temperature is a result of aggregation between BSM molecules. Given that aggregation between mucin molecules occurs through the interaction between the unglycosylated terminal domains [37,38], heat-induced increase in the hydrodynamic diameter of BSM is an indication that stronger alteration might have occurred to the globular terminal domains of BSM.

3.3. Influence of heat treatment on the adsorption behavior of BSM onto PDMS surface

The influence of thermal treatment on the surface adsorption behavior of BSM onto a PDMS surface has been studied. The adsorbed mass of an untreated sample was measured as a reference. Representative surface adsorption profiles of the BSM samples are presented in Supplementary Information (Fig. S2). The adsorbed mass for untreated BSM was found to be 185 ± 3.4 ng/cm² (Fig. 4). A slight increase was observed when BSM was heated to physiological temperature, 37 °C for 10 min, giving an adsorbed mass of 199 ± 4.9 ng/cm², followed by an adsorbed mass of 189 ± 8.5 ng/cm² when heated at 80 °C for 10 min. This value was comparable to that of untreated BSM. However, decrease in adsorption mass was observed after extended thermal treatment. After 30 min heating at 80 °C, an apparent decrease was observed with an adsorbed mass of 150 ± 18.8 ng/cm². Even further decrease was observed after 120 min of heating at 80 °C, from which the adsorbed mass was found to be 99 ± 10.4 ng/cm². Since the adsorption was allowed to room temperature after heating, the significant decrease in adsorption after extended thermal treatment is closely related to the aggregation of BSM (Fig. 3) addressed in the previous section under this condition, rather than the conformational changes at elevated temperatures (Fig. 1) and the consequent changes in the interaction of individual BSM molecules with the PDMS surface. The occurrence of aggregates on the surface would lower the overall packing density as each individual aggregate would take up a larger area on the surface. This inefficient packing onto the surface would subsequently lower the overall mass uptake of BSM. Nevertheless, the relationship between the size distribution of BSM molecules (Fig. 3) and the adsorbed masses (Fig. 4) is not simply inversely linear; BSM molecules cannot be modeled as a rigid sphere, they are polydisperse, and there are distinct surface interacting and non-interacting patches in BSM molecules.

3.4. Impact of thermal treatment on the lubricating properties of BSM

Lubrication in aqueous environments is one of the characteristic features of mucins [25,39,40]. In the present study, a MTM and a pin-on-disc tribometer were employed to characterize the lubricating properties of thermally treated BSM. PDMS was chosen as tribopair due to its compliance, the mechanical properties (elasticity modulus in the range of 1–2 MPa) being similar to those of biological tissues and facile fabrication into pin and disc shapes. The adsorption of amphiphilic BSM onto the PDMS surface confers hydrophilic properties [14], making it ideal for soft elastohydrodynamic lubrication [25]. Since MTM is equipped with a closed pot for lubricated tribo-contacts, it is feasible to control the temperature of the lubricant as well as to measure the friction forces during temperature variation. BSM was heated at increments of 10 °C from 5 °C to 85 °C, followed by the reverse measurements back to 5 °C, while measuring the friction forces at every step. MTM results are shown in Fig. 5. As can be seen in Fig. 5A, the BSM solution reveals a consistently low coefficient of friction of ca. 0.02 up to 35 °C (black, dark grey, grey and light grey lines). As mentioned above, this is a result of the facile adsorption of BSM onto PDMS in the low-temperature regime (Fig. 4). A slight increase was observed at 45 °C (cyan) to an average coefficient of friction of ca. 0.08 (Fig. 5A) followed by a ten-fold increase in coefficient of friction at 55 and 65 °C (dark yellow and blue), respectively, to ca. 0.2. At the highest temperatures, BSM exhibits a significant deterioration in lubrication capability compared to the measurements performed at physiological temperature or lower. Measurements at 75 °C and 85 °C show an average coefficient of friction of roughly 0.35 (Fig. 5A, green and red, respectively). As a reference, the coefficients of friction were obtained in the same temperature range from the sliding contacts between PDMS surfaces lubricated with PBS buffer solution only. The observed coefficients of friction were between 0.7 and 0.8, which were much higher than those obtained from the PDMS–PDMS sliding contacts lubricated with BSM solution (data not shown). No clear trend with the change of temperature was observed.

Since the tribological measurements were performed under boundary lubrication conditions, i.e. under low speed and high load, the lubricating efficacy is chiefly determined by the efficacy of adsorption of amphiphilic BSM onto the PDMS substrate. Thus, increase in friction forces at the higher temperatures could also be linked to many factors that deteriorate the efficacy of BSM adsorption at high temperatures. For instance, increasing temperature may reduce the resident time and binding stability of individual BSM molecules on the surface due to increased mobility at elevated temperatures. This effect, however, cannot be confirmed due to the lack of information on the adsorption and desorption of BSM molecules at elevated temperatures. It is noted though that the variation of the coefficient of friction as a function of temperature (Fig. 5) is strongly correlated to the change in the secondary structure of BSM as a function of temperature (Fig. 1), including their reversibility. Loosening of the random coil structure of BSM with increasing temperature may lead to non-optimal packing onto the surface due to the increase in size of individual BSM molecules.
molecules, and thus can be another contributing factor to the increased friction forces of BSM at elevated temperatures. With decreasing temperature, BSM started to regain its effective lubricity and the coefficient of friction decreased again. At the intermediate temperatures of 55°C and 65°C (olive and blue dashed lines, respectively), the coefficients of friction were observed to be somewhat lower than prior to heat treatment. At 55°C (dark yellow dashed line), the coefficients of friction were found to be comparable to those of the lower temperatures. At physiological temperature or lower, BSM fully regains its lubricity as the coefficients of friction measured for 35°C, 25°C, 15°C and 5°C (light grey, grey, dark grey and black dashed lines, respectively) are very close and deviating by less than 0.01 on average (Fig. 5A) compared to the values obtained prior to the high temperature measurements. By plotting the coefficient of friction against the temperature (Fig. 5B), it becomes even clearer that the variation in temperature clearly impacts the lubricity of BSM, but also that the effect is reversible. Furthermore, the exposure time does not appear to be an important factor as the coefficient of friction measured at the beginning (100 s), intermediate (300 s) and end (500 s) time-points produce similar coefficient of friction values. The inherent absence of any other secondary structural motifs than random coil structures as measured by CD spectroscopy may play a significant role in the reconstitution of the lubricity of BSM with lowering temperature. As the structure prior to heating had little higher degree of secondary structure to lose initially, the recurrence of the increased lubricity indicates that refolding into a random coiled secondary structure does not affect the tribological function of BSM.

The lubricating properties of BSM that are thermally treated and subsequently cooled to room temperature were also investigated by means of pin-on-disc tribometry. The samples were thermally treated in the same manner as for OWLS, i.e. at 37°C for 10 min or at 80°C for 10 min, 30 min or 120 min, respectively. The results (Fig. 6) show that extensive heating does not impact the lubrication properties of BSM. It was observed that BSM retained its lubricating ability and showed roughly the same coefficient of friction of 0.015–0.02 after the varied heat treatments. It is notable that decrease in the adsorbed mass for the samples heated at 80°C for 30 min and 120 min (Fig. 4) did not influence the lubricating properties of heated BSM samples. This is possibly because the absolute amount of adsorbed mass (99 ± 10.4 ng/cm²) is sufficiently high enough to provide effective lubrication even for the BSM samples heated at 80°C for 120 min, even though it represents the smallest adsorbed mass in a relative sense. The aggregation that occurred to BSM after extended heating at 80°C (Fig. 4), possibly via the interaction between C- and N-terminal regions, has not degraded the adsorption strength of BSM onto the PDMS surface, which is crucial for effective aqueous lubrication. Alternatively, it is rather possible that changes that occurred to the C- and N-terminal regions have strengthened the adsorption at the cost of lowering adsorbed mass as discussed above. Together with the friction values measured during the change of temperature presented in Fig. 5, the results indicate that the lubricating properties of BSM do not undergo any form of deterioration from extended thermal exposure.

4. Conclusions

In this work, the thermostability of BSM was investigated in terms of its structure and conformation in bulk solution, adsorption, and lubricating properties at an elastomeric hydrophobic PDMS surface. CD spectroscopy studies indicated that BSms display an overall random coil secondary structure. Upon gradual heating from 5°C to 85°C, the BSM structure was found to undergo appreciable changes with increasing temperature. These changes were, however, fully reversible when the temperature was lowered again. Extended thermal treatment at 80°C for up to 120 min was also found not to impact the secondary structure of BSM. In
addition, DLS studies showed that the hydrodynamic diameter of BSM slightly increased upon heating at 80 °C, which is interpreted as an indication of aggregation between BSM molecules. The adsorption of BSM onto a PDMS surface was shown to decrease after extended thermal treatment for 120 min at 80 °C. This could arise from an increase in aggregation between BSM molecules, causing less efficient packing onto the surface substrate. The lubricating properties of BSM showed a gradual deterioration with increasing temperature, and the coefficient of friction peaked at temperatures of 75 °C and above. Upon cooling, however, coefficients of friction similar to those observed prior to heating were reestablished. The deterioration of the lubricating capabilities of BSM at high temperature could be correlated to the conformational changes of BSM in bulk solution and the reduced adsorption onto the PDMS surface. However, high temperature treatment and subsequent cooling showed that BSM is heat-stable and retains its lubricating function even after extended periods of heating. The data presented here suggest that the overall lack of more complex secondary structural motifs in BSM, which is arising from heavy glycosylation of the central domain, is likely to contribute to the heat tolerance and therefore does not significantly impact on its functions. Since polymeric materials should be sterilized for biomedical applications, the ability to apply a mucin coating prior to sterilization would be advantageous and further increases the potential of BSM as a biocompatible surface coating material.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcis.2014.03.006.

References

Comparative studies of gastric and submaxillary mucins: Influence of pH on the conformation, surface adsorption and aqueous lubricating properties

Mucins are highly adapted to the environment in which they are expressed. In this study we were interested in comparing the adsorption and tribological behavior of two mucins, under neutral- and acidic conditions, which originate from two very different environments in the body.

My contributions to this paper were the purification of both BSM and PGM, CD spectroscopy measurements, adsorption measurements by OWLS, PoD tribological measurements and authoring the manuscript.
Comparative studies of gastric and submaxillary mucins: Influence of pH on the conformation, surface adsorption and aqueous lubricating properties

Jan Busk Madsen,1 Javier Sotres,2 Kirsi I. Pakkanen,1 Petr Efler,1 Thomas Arnebrant,2 Birte Svensson,3 Maher Abou Hachem,3 and Seunghwan Lee1

1Department of Mechanical Engineering, Technical University of Denmark
2Department of Biomedical Sciences, Faculty of Health and Society, Malmö University, 20506 Malmö, Sweden
3Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark

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Abstract

The properties of mucins from two different origins, namely bovine submaxillary mucin (BSM) and porcine gastric mucin (PGM), were investigated under neutral- and acidic conditions. Using circular dichroism (CD) spectroscopy, it was shown that no structural reorientation occurred within the mucins under either condition. However, differences between the mucins were recorded. While the far-UV spectra of PGM appears to display a fully random coil structure, BSM showed some structural similarity to that of a poly pro (II) helical conformation. This may be due to the differences in the oligosaccharides of the central domains. No tertiary structure was observed for either mucin. Emerging aggregation was recorded for BSM under acidic conditions using dynamic light scattering (DLS). This was also reflected in the surface adsorption properties where the surface mass was doubled. No changes were recorded for PGM. Quartz crystal microbalance dissociation (QCM-D) showed a less dense film formation on the surface for PGM when compared to BSM. This was mirrored in the tribological properties where both BSM and PGM displayed increased lubricity under acidic conditions. However, the lubricity of PGM failed in the low speed regime. This may be related to the poor film stability on the surface.
Introduction

Mucins are the major macromolecular component of mucus gels and the glycocalyx covering the epithelial lining. Structurally, mucins are determined to contain unglycosylated N- and C-terminal domains and a heavily glycosylated central domain. The major function of mucins is to act as a physical barrier that both protects the epithelium from abrasions and hinders potential pathogens from entering the tissue. Additionally, cell membrane-tethered mucins have also been shown to play a role in cell signaling via tyrosine phosphorylation pathways triggering, among other things, pathways involved in cellular motility and adhesion.

Apart from being a key component of mucus, mucins display various interesting surface and interfacial properties as polyanionic macromolecules, such as friction reduction, antibacterial coatings and as surface coating models for drug delivery systems. A previous study employing as-received PGM from a manufacturer (arPGM) reported that a superior lubricating effect is observed at acidic pH when compared to neutral pH conditions. It was attributed to the unique aggregation between arPGM molecules under acidic conditions and stabilization of PGM films on the surface. In fact, aggregating properties of gastric mucins have been verified by many techniques, such as rheometer, light scattering, fluorescence spectroscopy, and atomic force microscopy. A fundamental question addressed here is thus whether the acid-induced enhancement of the lubricating properties observed from arPGM would be observed from other mucins too. Two parameters are considered in this study. Firstly, we have investigated the influence of acidification on the lubricating properties of mucins by employing two different types of mucins from different biological origins, namely bovine submaxillary mucin (BSM) and porcine gastric mucin (PGM). While structural similarity of mucins from varying origins has been well recognized, fine differences in the structural and biophysical properties of
them have been studied to much less extent to date. As mucins are known to be highly adapted to the environment of their origin, and their general function in the organism, different structural features of the two mucins may lead to different responses in their lubricating properties due to changes in pH. Secondly, in order to establish genuine responses of the two mucins to pH changes, without interference by other commonly present non-mucinous biomolecules in commercial mucin samples, additional chromatographic purification was carried out. This provides an opportunity to compare the pH-responsive lubricating properties of mucins with or without other contaminants in the samples as well and showing meaningful differences between them.

Material and Methods

Mucins and buffers

BSM and PGM were purchased from Sigma Aldrich (Sigma Aldrich Brøndby, Denmark). BSM was purified as described in a previous study. Briefly, BSM was dissolved overnight at 4°C on a nutating mixer in 10 mM Na-acetate, 1 mM EDTA, pH 5.0 buffer to a final concentration of 10 mg/mL. The solution was clarified by sterile filtration (5 µm hydrophilic polyethersulfone sterile filter; Pall Corporation, Cornwall, U.K.) and subsequently fractionated according to their charge on a high load 16/26 Q Sepharose high performance anion exchange column (GE Healthcare Life Sciences, Uppsala, Sweden) installed on an Äkta Avant Chromatograph (GE Healthcare, Uppsala, Sweden). Proteins bound on the column were fractionated with a multi-step gradient of high salt elution buffer (10 mM Na-acetate, 1 mM EDTA, 1.2 M NaCl, pH 5.0). Fractions containing protein were analyzed by SDS-PAGE and stained using coomassie brilliant blue to visualize proteins or periodic acid/Schiff staining to identify fractions with glycoproteins. BSM fractions were pooled, dialyzed against milliQ grade water and subsequently freeze-dried. All steps were performed at 4°C to minimize potential proteolytic degradation.
PGM was purified using the same system as described above. However, due to PGM’s tendency to form aggregates, additional sterile filtration was performed. PGM was filtered through 5 µm sterile filters followed by 1.2 µm sterile filters (hydrophilic polyethersulfone sterile filters; Pall Corporation, Cornwall, U.K.), three times each, to clarify the solution. Fractions containing protein were analyzed by SDS-PAGE and coomassie brilliant blue staining, pooled, dialyzed against milliQ grade water and subsequently freeze-dried. The chromatogram and SDS-PAGE analysis of PGM can be found in the supplementary information (Figure S1). All samples were stored at -20 °C and desiccated prior to use.

Due to the wide difference in the pHs employed in the present work, a citrate-phosphate buffer system was chosen. Citrate-phosphate buffer at pH 2.4 (0.01 M citrate / 0.001 M phosphate) and at pH 7.4 (0.0001 M citrate / 0.02 M phosphate) were prepared and NaCl to a physiological concentration of 150 mM was added. BSM and PGM samples were dissolved in either buffer prior to use on a nutating mixer to a final concentration of 1 mg/mL. All chemicals used in this study were of laboratory grade and purchased from Sigma Aldrich (St. Louis, MO).

**Circular Dichroism (CD) Spectroscopy**

Circular dichroism (CD) spectra of BSM and PGM solutions were acquired by employing a rectangular quartz cuvette with 0.5 mm path length for far UV measurements and a 10 mm rectangular quartz cuvette for near UV measurements (Hellma GmbH & Co. KG, Müllheim, Germany) using Chirascan spectrophotometer (Applied Photophysics Ltd., Surrey, UK). Due to signal interference, especially in the far UV spectrum, the buffer solutions were diluted 1:2 with milliQ grade water. The pHs of the diluted buffers were measured to be pH ~3 and 7.4, respectively. Far UV spectra were recorded in the region from 240 to 195 nm with a step size of 1 nm, bandwidth of 1 nm, and time-per-point value of 1.5 s. Near UV spectra were recorded from 400
to 260 nm (to 240 nm for arPGM) with a step size of 1 nm, bandwidth of 1 nm, and a time-per-point value of 0.5 s. One spectrum was obtained from the averaging of three traces. All samples were measured in triplicate and averaged again, and any background signal from buffer was subtracted.

**Dynamic Light Scattering (DLS)**

Samples were prepared in 0.22 µm-filtered buffers and measured using a Malvern Zetasizer Nano ZS two angle particle and molecular size analyzer (Malvern Instruments, Worcestershire, UK). Non-invasive back scatter (NIBS, measurement angle 173 °) was used to give the highest sensitivity. The light source is a He-Ne laser at 633 nm and the temperature was set at 25 °C. Disposable cuvettes (PMMA, Plastibrand™) were employed for DLS measurements. Each sample was measured in triplicates. The Malvern Zetasizer software (Version 7.02) was used to analyze the obtained data. All DLS data were plotted according to the intensity distribution of the hydrodynamic diameter ($D_h$).

**Optical Waveguide Lightmode Spectroscopy (OWLS)**

OWLS is based on grating-assisted in-coupling of a He-Ne laser into a planar waveguide coating (200-nm thick Si$_{0.25}$Ti$_{0.75}$O$_2$ waveguiding layer on 1 mm thick AF 45 glass (Microvacuum Ltd, Budapest, Hungary)). Adsorption of biomolecules from bulk liquid to the interfacing solid surface is measured by monitoring the changes in the refractive index at the vicinity of the solid-liquid interface. This method is highly sensitive out to a distance of ~200 nm from the surface of the waveguide. Experiments were carried out using the OWLS 210 Label-free Biosensor system (Microvacuum Ltd, Budapest, Hungary).

In order to keep the substrates for surface adsorption studies by OWLS and the tribology studies (see below) consistent, waveguides were coated with a layer of poly(dimethylsiloxane) (PDMS).
The waveguides were spin-coated at 2500 rpm for 15 s initially with an ultrathin layer (ca. 24.3 ± 3.1 nm, determined by scratch test using atomic force microscopy in tapping mode) of polystyrene (Sigma Aldrich, St. Louis, MO) dissolved in HPLC grade toluene at 6 mg/mL. The base and curing agent of a commercial silicone elastomer (Sylgard 184 elastomer kit, Dow Corning, Midland, MI, USA) were dissolved in hexane at a ratio of 10:3 (final concentration, 0.5 % w/w). A subsequent ultrathin layer of PDMS (ca. 16.4±0.17 nm) was then added by spin-coating at 2000 rpm for 25 s and lastly cured in an oven at 70 °C overnight.

The PDMS-coated waveguide was exposed to the appropriate buffer prior to sample injection until a stable baseline was obtained. A programmable syringe pump (Model 1000-NE, New Era Pump Systems, Inc., NY) was used to pump buffer solutions through a flow-cell over the OWLS waveguide surface. 100 µL of sample was then injected via a loading loop. Upon observing surface adsorption, the pump was stopped so that the BSM or PGM molecules could adsorb onto the surface under static conditions. After 10 min, the flow cell was rinsed with the appropriate buffer by restarting pumping. The adsorbed mass density data were calculated according to de Feijter’s equations. The experiment was repeated two or three times for each mucin at either pH. A refractive index increment (dn/dc) value of 0.150 cm³/g for BSM was used for the calculation of the adsorption masses.

Quartz Crystal Microbalance with Dissipation (QCM-D)

QCM-D measurements were performed by using an E4 system (Q-Sense AB, Sweden). A detailed description of the technique and its basic principles can be found elsewhere. Gold sensors (Q-sense AB, Sweden) coated with PDMS by the spin-coating technique were used (see the section for OWLS for details). Solutions were supplied into the QCM-D chamber using an Ismatec peristaltic pump IPC-N 4 at a flow rate of 0.2 mL·min⁻¹. Protein-free buffer was first injected into
the chamber until a stable baseline was observed. Then, protein solution was flowed through the chamber until adsorption was observed. At this point the flow was stopped and the protein left to adsorb for 10 min under non-flow conditions. Finally, the cell was rinsed for 5 min with the corresponding protein-free buffer solution and then stabilized for ca. 10 min under non-flow conditions. Each experiment was performed twice. During the experiments, shifts in frequency, $\Delta f$, and dissipation factor, $\Delta D$, for the different overtones (n=3, 5, 7, 9, and 11, even though only data for n=3 is presented in the manuscript) were monitored.

**Pin-on-Disc (PoD) Tribometry**

The influence of pH on the lubricating properties of BSM and PGM was investigated using a pin-on-disc tribometer (CSM, Peseux, Switzerland). This approach is based on a loaded pin forming a sliding contact with a disc. The load on the pin was controlled by the application of deadweight (1 N). The friction forces between them were measured at incrementing speeds of the disc from 0.25 mm/s to 100 mm/s. Thus, the coefficient of friction ($\mu$) vs. speed plots was acquired. Disc rotation was enabled by a motor beneath the disc while the pin remained stationary. Friction generated during sliding contacts was monitored by a strain gauge. Friction forces data were acquired over 20 rotations at a fixed radius of 5 mm. A PDMS tribopair was used for the experiments. PDMS was prepared by thoroughly mixing base fluid and crosslinker of a Sylgard 184 elastomer kit (Dow Corning, Midland, MI) at a ratio of 10:1. Gentle vacuum was applied to remove air bubbles generated during mixing. The discs were prepared by casting the PDMS mixture into a home-machined aluminum plate with flat wells designed to the dimensions (30 mm diameter $\times$ 5 mm thickness) of the tribometer. A 96 microwell plate (NUNCION Delta Surface, Roskilde, Denmark) with a hemispherical end ($\varnothing = 6$ mm) was used as a mold for casting the pin. The PDMS mixtures were cured at 70 °C overnight.14
Results and Discussion

pH impact on the structural conformation of BSM and PGM

The secondary and tertiary structural conformations of BSM and PGM and their changes by pH were investigated by CD spectroscopy at pH ~3 and 7.4 (Figure 1). Due to interference from the chosen buffer system at pH 2.4, the pH was raised to ~3 where no interference in the spectra was observed. The subsequent data acquired in this study was all done at pH 2.4 and pH 7.4.
Figure 1 A) Far-UV measurements of BSM and PGM in the range from 260 nm to 195 nm. BSM pH 7.4 and BSM pH ~3 are indicated in black and grey, respectively, while PGM pH 7.4 and PGM pH ~3 are red and magenta. B) Near-UV spectra recorded for BSM and PGM. Measurements are
indicated as in A). C) Near-UV spectra recorded for arPGM. The signal for arPGM pH 7.4 has been colored orange while arPGM pH 2.4 is blue. The inset shows the far-UV measurements for arPGM.

The far-UV spectra showed that both BSM and PGM lacked distinct and well defined secondary structures, such as $\alpha$-helices, $\beta$-sheets, or $\beta$-turns. This is not surprising as most of the mucin structure is made up of the heavily glycosylated PTS repeat central domains. Only the terminal N- and C-terminal domains have been assigned to contain structural motifs determined from the peptide sequence including cysteine knots, CysD domains and Von Willebrand factor binding domains that are in involved in mucin-mucin interactions. However, seeing as the vast majority of the molecule consists of the glycosylated central domain, the signal contribution from secondary structures present in the N- and C-terminal domains will be vastly diminished. The far UV CD spectra of BSM show a slight local positive maximum at ca. 218 nm and a large negative minimum at ca. 201 nm. On the other hand, PGM shows only a single large negative minimum at ca. 207 nm (Figure 1A). The features presented by BSM in the far UV range is quite similar to that of poly(Pro) II helices, even though the exact peak positions are somewhat shifted from those observed from poly(Pro) II peptides (where the major peak minimum is observed at 196 nm). Systematic studies with well-prepared poly(Pro) II peptides have shown that polypeptides showing the signals in this region are not entirely unordered, but in fact possess helices arising from proline residues. With the presence of a significant number of proline amino acid residues in the primary structure of BSM, it is not unreasonable that secondary features pertaining to the high concentration proline residues is present in the far-UV spectra. It is rather intriguing, however, that the far-UV CD spectra of PGM differ from that of BSM and do not display features of poly (Pro) II helical motifs, despite also containing central domains made up of the glycosylated tandem repeat PTS-domains. A fundamental difference between mucins originates in the structure of the $O$-linked
glycosylation moieties attached to the central domains of the proteins.\textsuperscript{35-37} Thus, the variation in the carbohydrate structures may explain the difference observed in the signal from BSM and PGM in their far-UV CD spectra. Both the density and especially the abundance of negatively charged carbohydrate moieties in the oligosaccharides attached to BSM (\textasciitilde30\% of total carbohydrate mass is sialic acid) appear to be much more substantial than in PGM (2-9\% sulphate terminal group modifications).\textsuperscript{35-39} As the most abundant oligosaccharides that are attached to the central domains of BSM are made up of three carbohydrate residues, sialic acid is most likely evenly distributed throughout. The presence of a highly negatively charged group in the vicinity of every third amino acid residue imposes further steric constraints on the $\phi$ and $\psi$ torsion angles in the polypeptide backbone than was already imposed by the cyclic side chain of proline.\textsuperscript{40} In comparison the lesser charge density in the central domains of PGM offer more rotational freedom in the apoprotein. It is therefore likely that intramolecular repulsion between the negatively charged oligosaccharides force BSM into a more ordered structure in comparison to the lesser charged PGM.\textsuperscript{35-40}

For both BSM and PGM, pH does not seem to affect the far-UV CD spectra, except for the slightly reduced negative peak intensity by ca. 5 millidegrees for PGM at acidic pH. A potentially expected structural change from lowering pH from 7.4 to \textasciitilde3 is related to the protonation of carboxylic groups in glutamic- and aspartic acids (pKa \approx 4) present in polypeptide backbone as well as histidine residues (pKa \approx 6). On the other hand, the pKa of sialic acid has been determined to be 2.6\textsuperscript{41} while the pKa of sulphate groups are closer to \textasciitilde1.\textsuperscript{42} Thus, the overall charge of the proteins is most likely retained and the protonation of the amino acid moieties are inconsequential for the overall structural integrity. Nevertheless, as the majority of the far-UV CD signals originate from the structure of the glycosylated central PTS-domains, it is not possible to verify whether the pH change causes structural changes in the N- and C-terminal domains. This may potentially lead to structural rearrangements in the folding of the structural motifs known to be present in the N- and C-terminal
parts of the protein and partly explain the increase in hydrodynamic size, surface adsorption and the lowering of the friction coefficient described below.

Differences in the tertiary fingerprint of BSM and PGM were also investigated by near-UV CD (Figure 1B). In the near-UV spectrum, the signal arises from vibrations and reorientation of aromatic amino acid residues and also disulfide bonds. Changes measured in the near-UV signal can be interpreted to originate from either aggregation or higher structural organisation in the tertiary structure of proteins.\textsuperscript{26-27} Figure 1B show the near-UV CD signal recorded for BSM and PGM under acidic and neutral conditions. Absence of signal in 270 to 400 nm region indicates that there is no tertiary structure for the two mucins at pH 7.4, and no structural change is induced by changing pH from 7.4 to ~3. A previous study of BSM also showed no signal for BSM in another neutral aqueous buffer system,\textsuperscript{20} which is consistent with the present study. Furthermore, no pronounced signal in the near UV CD spectra from arBSM was recorded at either neutral or acidic pH (data not shown). arPGM, on the other hand, has been shown to contain higher ordered structure as measured by near-UV CD spectroscopy in a previous study.\textsuperscript{14} As a control, far- and near-UV CD spectra of arPGM were measured (Figure 1C). While the far-UV CD spectra are nearly identical to those for purified PGM (Figure 1C, inset), the near-UV CD spectra differed substantially. An intense signal was recorded for arPGM at pH 7.4 with a local maximum at ca. 270 nm (Figure 1C). The signal at low pH showed a diminished signal with a maximum intensity of that of the sample in neutral pH. Lee et al.\textsuperscript{14} interpreted the change in the signal from neutral to low pH as partial unfolding of the tertiary structure of arPGM. The near-UV signal recorded from arPGM may potentially originate from two sources. Firstly, commercial mucins are known to contain varying quantities of non-mucin proteins that may be completely responsible for the occurrence of tertiary structure signal in the arPGM. Secondly, our experiences have shown that PGM aggregates
extensively in solution and the signal could potentially originate from these aggregates (supplementary figure S3). The additional purification step by anion exchange chromatography greatly reduced the presence of both species in the sample (supplementary figure S2 and S3).\textsuperscript{20}

**Impact of pH on the hydrodynamic size of mucins**

Figure 2 shows the size distribution of BSM and PGM according to intensity at pHs 2.4 and 7.4.

![DLS measurements of BSM and PGM](image)

Figure 2 DLS measurements of BSM (pH 7.4, black and 2.4, grey) and PGM (pH 7.4, red and 2.4, magenta) at neutral and acidic pH.

At pH 7.4, the maximum peak positions for BSM and PGM were determined to be 60 nm and 55 nm, respectively. Common for both mucins is that no molecular species with a hydrodynamic diameter larger than 300 nm are present at pH 7.4. Upon lowering pH to 2.4, the maximum peak positions shifted to 77 nm for BSM and 90 nm for PGM, respectively. BSM additionally displayed emerging formation of aggregates as peak broadening with species between 200 nm and 1000 nm starts to appear. PGM on the other hand, did not show formation of any larger species, indicating
that no aggregates had started to form at pH 2.4. Previous studies have suggested that acid-induced aggregation of PGM is mainly driven by hydrophobic interactions between the unglycosylated regions in the proteins.\textsuperscript{16} The lack of aggregation occurring between PGMs at low pH in this study may be due to the manufacturer’s purification process. It has previously been reported that it uses harsh conditions that denature the protein, and our subsequent additional purification may potentially be selective towards the non-aggregating PGMs.\textsuperscript{43-44} At pH 2.4, the environment is more acidic than the pKa of the sialic acid in the oligosaccharides of BSM (pKa = 2.6). As it is only slightly below the pKa, the molecule is most likely not fully protonated. However, the protonation of the charged species would decrease intra- and intermolecular repulsion between the mucins in solution and thus explain the emergent aggregation of BSM.

**Surface adsorption: OWLS**

OWLS was employed to monitor the surface adsorption behaviour of the mucins at the different pHs. The results are presented in Figure 4.
Figure 3 OWLS measurements showing the adsorbed masses of BSM and PGM and neutral and acidic pH.

At neutral pH, the amount adsorbed to the hydrophobic surface was 54 ± 2.4 ng/cm² for BSM and 87 ± 1.3 ng/cm² for PGM (Figure 3) after 10 minutes of surface exposure. Lowering the pH significantly increased the amount of adsorbed BSM to 116 ± 1.1 ng/cm² while a slight decrease to 77 ± 0.1 ng/cm² was observed for the adsorption of PGM (Figure 3). A previous study of BSM adsorption onto hydrophobized silica surfaces using a less acidic buffer (pH 3.8, 2 mg/mL) showed an increase in the surface adsorption too. The increase of adsorption of BSM at acidic pH in the present work is most likely due to that the intermolecular electrostatic repulsions are diminished as the pH approaches the isoelectric point (IEP) of BSM (pH 3) and the protonation of the sialic acids in the oligosaccharides. This allows for more efficient interaction between BSM molecules as well as the interaction between BSM and the PDMS substrate surface, as the repulsion between the overall negative charge of BSM and the nonpolar surface is suppressed. In fact, this is a typical behaviour expected from the adsorption of amphiphilic polyanions onto nonpolar surfaces from aqueous solution. As the electrostatic repulsion between neighbouring BSM molecules on the surface occurs in a more confined space than in bulk solution, the increase in adsorbed mass (Figure 3) appears to be more enhanced than the size distribution (Figure 2).

PGM, on the other hand, showed much less sensitive response to pH change as only a slight decrease in the adsorbed mass (by 11%) was observed at pH 2.4 compared to that at pH 7.4. In a previous study, decrease in adsorbed mass of arPGM at low pH was attributed to the increase in the average hydrodynamic size of arPGM observed by DLS. The PGM aggregates would essentially take up a larger area on the hydrophobic surface, causing a decrease in the packing density onto the surface. The magnitude of decrease in the adsorbed mass of PGM by acidification in this study is,
however, much smaller than that for arPGM, presumably because the aggregation at low pH for the purified PGM is less extended as shown in Figure 2. The lack of increase in the adsorbed mass at low pH, as with the case of BSM, indicates that PGM has less charged moieties and/or the electrostatic repulsion on the surface is less significant even at pH 7.4.

Surface adsorption: QCM-D

In a QCM-D experiment instrument, a PDMS-coated quartz sensor is oscillated by applying an alternating-current voltage across it. The raw experimental data provided by QCM-D consist of shifts in the resonance frequencies of the sensor, $\Delta f_n$ where $n$ is the overtone number, and in the dissipation factor, $\Delta D_n$, which is proportional to the ratio between the dissipated and the stored energy during a single oscillation. If material is adsorbed on the sensor, the resonance frequencies decrease. If the adsorbed material forms a rigid elastic film, the frequency shift is linearly related to the adsorbed mass by means of the Sauerbrey equation. However, if the adsorbed film has a marked viscoelastic character, i.e. high shift in dissipation factor, the Sauerbrey approach underestimates the adsorbed mass. With respect to $\Delta D_n$, a high value indicates that it loses energy quickly, suggesting a viscous character for the adsorbed film. A low value would suggest a rigid elastic character for the adsorbed film instead.

In this work, QCM-D was used to study the adsorption of BSM and PGM molecules on PDMS surfaces both at pH 7.4 and pH 2.4 (Figure 5, only data for the $n = 3$ overtone are plotted for simplicity). First, clear differences were observed between the adsorbed BSM and PGM. Independently of the solution pH, PGM led to films characterized by higher shifts in frequency and dissipation both before and after being rinsed with protein-free buffer. A higher shift in frequency suggests a higher mass coupled to the sensor. QCM-D is sensitive to the solid components adsorbed on the sensor (mucins) as well as to the solvent coupled to the sensor. In fact, it has been shown
that for adsorbed mucin films that the coupled solvent is the main contributor to the QCM-D response \(^{52}\). Thus, \(\Delta f\) data suggest that PGM films have a higher ability for coupling the surrounding solvent than BSM films. With respect to \(\Delta D\) data, the higher values observed for PGM films suggest that they have a higher viscous character than BSM films.

BSM and PGM films present another difference observed during the adsorption process. In the case of BSM films, an initial overshoot, mostly in the dissipation shift but also slightly in the frequency shift, is observed, and is followed by a gradual decrease at both pHs. This is well illustrated in the \(\Delta D\) vs \(-\Delta f\) plot in Fig. 5c. This behaviour, which has previously been observed for BSM films formed on substrates of hydrophobic nature,\(^{52-54}\) can be explained by the BSM films capturing a high amount of solvent in the beginning of the adsorption process, which is then gradually released as the BSM film goes through a conformational change. The incorporation of a large amount of solvent in the beginning is ascribed to the amphiphilic character and the strong hydration capabilities of BSM molecules adsorbed on hydrophobic surfaces. Interestingly, PGM films did not exhibit a similar behaviour (Fig. 5d). An almost linear relation between \(\Delta D\) and \(\Delta f\) was observed instead.
The QCM-D results revealed a similar influence from the environmental pH on the two types of mucin films. In both cases, the shifts in frequency were higher at pH 2.4 than at pH 7.4, indicating that the “wet mass” coupled to the sensors was higher at the acidic condition. In contrast, shifts in dissipation were lower at pH 2.4 than at pH 7.4, suggesting that both mucin films were more rigid and elastic at acidic conditions. It is interesting to compare these results with those from OWLS.
BSM exhibited a higher “dry mass” at pH 2.4 than at pH 7.4. Thus, it is reasonable to speculate that this would be the origin for the associated increase in “wet mass”. In contrast, considering that the “dry mass” of PGM as characterized with OWLS (Figure 4) at pH 2.4 was slightly lower than at pH 7.4, it seems that the major influence of acidification for PGM films is to incorporate a larger amount of solvent. Interestingly, and counterintuitively, this is associated with lower ΔD values, i.e. lower viscous character.

**Lubrication**

Figure 5 shows the lubrication properties of BSM and PGM at neutral and acidic pH.

![Figure 5](image)

Figure 5 \(\mu\) vs. speed plot of self-mated sliding of PDMS surfaces lubricated with either 1 mg/mL of BSM (pH 2.4; grey squares, pH 7.4; black squares) or PGM (pH 2.4; magenta triangles, pH 7.4; red triangles) or buffer (green).
At neutral pH, the lubrication of the hydrophobic PDMS surfaces by the BSM solution is very effective with $\mu$ values between 0.02 and 0.07 in the speed range of 0.25 – 100 mm/s (Figure 5, black squares). The lubrication properties of BSM improved under acidic conditions where the $\mu$ values are lowered to between 0.003 – 0.01 in the same speed range (Figure 5, grey squares). As mentioned in the discussion of the adsorption behaviour of BSM above, the overall surface charge distribution of the molecule is diminished as the solution is close to the isoelectric point of the macromolecule and the sialic acids are protonated. Thus, thicker and more stable lubricating films formed at acidic pH can explain the improvement of the lubrication properties of BSM. This is also consistent with a recent study of antiwear properties of BSM on hydrophobized silica surfaces.\textsuperscript{45} Even though a larger “wet mass”, indebted from a larger amount of solvent, was observed at pH 7.4, relatively inferior lubricating capabilities at neutral pH suggests that these water molecules are weakly incorporated into the BSM film, and do not contribute to lubrication very effectively.

The lubricating behaviour of the PGM solution was observed to be nearly negligible at neutral pH. A slightly reduced $\mu$ values compared to buffer solution was shown at high speeds, such as 50 – 100 mm/s, but this is mainly due to the enhanced entrainment of buffer solution in the high-speed regime.\textsuperscript{55} In the low speed regime, where boundary lubrication is important, it becomes evident that there is no appreciable lubricating effect by the PGM film on the PDMS surfaces as the $\mu$ values are close to those measured for the buffer solution. It is emphasized again that the poor boundary lubricating properties of PGM is not resulting from poor adsorption properties on the PDMS surfaces, as higher amounts of PGM are adsorbed onto the PDMS surface at both pHs when compared to BSM at pH 7.4. Instead, it may rather be related to poor stability of PGM films on the PDMS surface. This is consistent with a previous study with arPGM solution (1 mg/mL) showing basically the same pH dependence in the lubricating properties at the PDMS-PDMS contact.\textsuperscript{14} In that study, the poor lubricating properties of arPGM solution at pH 7, despite a significant amount
of adsorbed mass (> 250 ng/cm² when the ionic strength is ca. 100 mM), was attributed to the weak binding strength of individual arPGM molecules on PDMS surface and easy removal of arPGM films by tribostress. The same argument can be applicable to the purified PGM in this study. Importantly, this view is supported by the QCM-D experiments in this study, where PGM films are much more viscous and loose in the conformation compared to BSM films (Figure 4).

At acidic pH, enhanced lubricity of the PGM solution was observed. The acid-induced improvement in the lubricating properties of PGM can be attributed to the enhanced stability of the lubricating films, as with arPGM. However, the μ values obtained by the PGM solution are still much higher than those by BSM, even at pH 2.4. This is somewhat different from the previous study where fairly effective lubricating properties (μ ≈ 0.04) were observed from arPGM solution up to the ionic strength of ca. 100 mM. The major difference can be assigned to the larger species (> 300 nm in hydrodynamic diameter) that were removed in the additional purification step for PGM. In fact, the aggregation induced by acid for the purified PGM is not as enhanced as expected from other studies, and it is consistent with only marginally improved lubricating properties of the PGM solution at low pH.

The exact origin of the formation of more viscous and loose films by PGM on PDMS surfaces compared to BSM is presently unclear. However, the lubricating properties, especially in the low-speed regime, provide important clues on the adsorption strength of mucin molecules on the PDMS surface, as it determines the efficacy of the boundary lubrication. From this study, it is clear that BSM can form a stable binding onto hydrophobic surface very effectively, and it can be explained by its distinct amphiphilicity. In turn, this can be explained by easy access and/or energetically favourable interaction of the “unglycosylated” regions of BSM molecules with hydrophobic surfaces, as they act as anchoring groups in the adsorption process. Likewise, poor lubricity of PGM, originating from the weaker binding onto the PDMS surface, may indicate that the interaction
of the unglycosylated regions of the PGM molecules with the hydrophobic surface is not sufficiently favourable. In turn, this could be because the length/molecular weight of the unglycosylated regions of PGM are short/small or unfolding of the polypeptide region in the interaction with hydrophobic surface is not favourable. Alternatively, the difference in binding strength can also be related to the difference in glycan structure of the two mucins. As mentioned above, PGM is known to contain far less negative terminal moieties in the oligosaccharide structures compared to BSM.

Conclusions

In this work, we have studied whether the formerly reported acid-induced aggregation and enhanced lubrication of arPGM\textsuperscript{14} would also be observed from two types of mucins with different biological origin. Additional purification was employed to remove the majority of non-mucin proteins from PGM and BSM. A variety of analytical tools, including CD spectroscopy for conformational changes of proteins, DLS for hydrodynamic size distribution, OWLS and QCM-D for surface adsorption properties, have been employed to investigate the structural and conformational features of the two mucins in bulk solution and on the PDMS surface. The acidic solution provided an environment where both mucins showed improved aqueous lubricating properties compared to the neutral aqueous solution. This is related to the polyanionic nature of mucins, and is ascribed to protonation of the many charged species of the mucins in the acidic environment which in turn enhances the favourable adsorption and formation of stable lubricating films on nonpolar PDMS surfaces. Interestingly, this effect was apparent for BSM only. Despite relatively more effective lubricating properties at acidic pH, the efficacy of PGM is inferior to that of BSM at both pH conditions. This is not a result of lower mass adsorption, but rather from the loose and viscous
characteristics of PGM films with a large amount of incorporated water. Consequently, PGM films cannot effectively withstand the tribological stress imposed on them.
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Supplementary information

Figure S1 Chromatogram of the elution profile of PGM with the salt gradient indicated (red). The bars indicate analyzed regions in Figure S2.
Figure S2 CBB stained SDS-PAGE analysis of fractions collected during anion exchange chromatography purification. The bars indicated I, II and III show fractions from the corresponding peaks in Figure S1. Peak III was selected for downstream applications.
Figure S3 Dynamic light scattering analysis of arPGM.
Proteolytic degradation of the terminal domains of bovine submaxillary mucin (BSM) and its impact on adsorption and lubrication

The overall tertiary structure of mucins are usually described as adopting a “dumbbell” like conformation with the globular domains separated by the glycosylated central domains of the protein. The amphiphilic nature of the protein dictates that the hydrophobic terminal domains of the molecule are responsible for adsorption onto hydrophobic surfaces. In this study, we were interested in investigating how the loss of the terminal domains impacts the adsorption and tribological properties of BSM.

My contributions to the work presented in this manuscript was purification of BSM, proteolytic digestion of BSM, SDS-PAGE analysis, size exclusion chromatography, Cd spectroscopy, Dynamic light scattering measurements, OWLS measurements, PoD measurements and authoring the manuscript.
Proteolytic degradation of the terminal domains of bovine submaxillary mucin (BSM) and its impact on adsorption and lubrication at the hydrophobic surface

Jan Busk Madsen,¹ Petr Efler,¹ Birte Svensson,² Maher Abou Hachem,² and Seunghwan Lee¹

¹Department of Mechanical Engineering, Section for Materials and Surface Engineering, Technical University of Denmark
²Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark

KEYWORDS: bovine submaxillary mucin (BSM), Proteolytic degradation, Surface adsorption, Lubrication
Abstract

The effect of proteolytic digestion on bovine submaxillary mucin (BSM) was investigated in terms of changes in its size, the secondary structure, surface adsorption and lubricating properties after treatment with two proteases that have distinctly different cleavage sites, namely trypsin and pepsin. SDS-PAGE analysis using two staining methods showed that only regions that were not glycosylated were degraded by the proteases. Size exclusion chromatography (SEC) and dynamic light scattering (DLS) showed that aggregates of the BSMs formed after proteolytic digestion. Changes to the secondary structure were investigated using circular dichroism (CD) spectroscopy and showed a slight reduction and shift in signal intensity owing to the removed terminal domains although the overall random coil structural conformation stayed intact. Using optical waveguide lightmode spectroscopy, the surface adsorption properties were determined. Tryptic digestion of BSM reduced the adsorbed mass significantly (roughly 13 ng/cm² vs. 43 ng/cm² for whole BSM). Pepsin digestion of BSM on the other hand showed a slight increase in the overall adsorption (53 ng/cm²) compared to whole BSM. Pin on disc (PoD) tribometry was used to assess the influence of the proteolytic digestions on the lubricating properties of the BSMs. The protease treatment of BSM completely degraded the effective lubrication properties of intact BSM. The data presented in this paper supports the notion that the hydrophobic terminal domains of BSM are integral to the hydrophobic surface adsorption and lubricating properties of BSM.

Introduction

Mucins are a family of large proteins that are either secreted or cell surface tethered and are a key component in the mucus gels that coat the epithelial lining. Here they act as a passive barrier that protects the underlying tissue from physical abrasion and bacterial infection [1, 2]. Secreted mucins
are produced in specialized goblet cells that are incorporated into the epithelial lining or in submucosal glands [3]. In the specialized cells they are stored as preformed mucin in a dehydrated form within granules [3-6]. The secreted mucins usually form polymerized complexes [7]. Secretion occurs either constitutively or as a response to extracellular challenges which ensures a rapid response during physical abrasion or bacterial adhesion [5]. Mucins are large amphiphilic proteins where the major part of the macromolecule consists of one or several central domains that are heavily modified with mostly O-linked oligosaccharides (Figure 1) [7, 8]. Due to the native charge distribution of the carbohydrates, the central domains carry an overall negative charge at physiological conditions. The N- and C-terminal domains are usually described as being globular with an overall neutral charge due to a high content of uncharged amino acid residues. The primary structure of the apoprotein part of the central domains is made up of a variable number of tandem repeats (VNTR) consisting of proline-, serine- and threonine amino acid residues. Due to their compositions and presence in all mucins, they have been termed mucin like PTS-domains [9-11]. Oligosaccharides moieties are attached via the linkage carbohydrate N-acetylgalactosamine (GalNac) to the VNTR’s on either the hydroxyl group of serine or threonine (Figure 1).

Figure 1 Conventional schematic representation of the secreted mucin such as BSM. Globular domains are found in the N- and C-terminal regions of the macromolecule and are separated by heavily glycosylated central domains and Cys domains.
The inset shows typical oligosaccharide structures found O-linked to the central domain of mucins. More information on the oligosaccharide structures can be found in [12-15].

The abundant attachment of oligosaccharides to the central mucin-like domains increases the overall size of the macromolecule greatly by 2-3 times that of the naked apoprotein [16]. Oligosaccharides are reported to constitute between 50-80% of the total weight of the macromolecule, while the difference in the carbohydrate branching structures contributes to the diversity found in mucins [1, 12-15, 17]. The most abundant oligosaccharides that are attached to the mucin apoprotein are either dimers or trimers of carbohydrates. However, even larger branched carbohydrate structures are interspersed throughout the central domain. Branched oligosaccharides containing up to six different carbohydrates have been identified to be present in bovine submaxillary mucin (BSM) [18] and porcine gastric mucin (PGM) [13]. Most of the smaller oligosaccharides are terminated by negatively charged groups such as the carboxyl group in sialic acid (pKa ~2.6) and sulphate groups (pKa ~1), rendering the overall charge distribution of the molecule to be highly negative under physiological conditions [19-21]. Analysis of BSM showed that up to 30% of the carbohydrates found on BSM are sialic acids [22] while up to 9% of the oligosaccharides found in PGM are sulphated [20]. The negative charge of the oligosaccharides is responsible for the intramolecular repulsion rendering the mucin to undertake their expanded “bottlebrush” like conformation depicted in Figure 1. An additional feature of the oligosaccharides is their ability to recruit water molecules from the surrounding environment due to the high concentration of hydrogen bonding groups on the surface, thus conferring hydrophilic properties to the macromolecule.

Mucins are usually described as having a “dumbbell” like structure as depicted in Figure 1 and act more like a rigid polymer than a protein when adhering onto a surface [23, 24]. When adsorbing onto a hydrophobic surface, the hydrophobic terminal domains are thought to interact with the surface while the charged central domains protrude away from the surface and into solution. In the
study presented in this paper we were interested in investigating how the loss of the terminal domains impacts the adsorption and tribological properties of BSM. It is generally accepted that the hydrophobic terminal domains are responsible for adhesion to the surface. By employing proteases we sought to degrade the terminal domains while keeping the bulk of the protein intact. As described above the central domains consist mostly of VNTRs and should therefore not contain cleavage sites. Furthermore, the extensive posttranslational oligosaccharide modifications of the central domains most likely hinder the access to any cleavage sites that may be present in the central domains due to their bulk blocking access to the apoprotein. To verify whether any of the changes we observed was an artifact of the specific protease that was employed, we tested the hypothesis that the terminal domains are integral for hydrophobic surface adsorption by using two commonly used proteases, namely trypsin and pepsin.

Materials and Methods

Proteins and chemicals

BSM (M3895-1 G, Type I-S, Lot. Nr. 039K7003V), pepsin, trypsin and neuraminidase along with all chemicals used for buffers etc. were purchased from Sigma Aldrich (Brøndby, Denmark).

Purification of BSM

Due to the known content of additional non-mucin proteins present in commercial BSM, an additional one-column anion exchange purification strategy was employed which is described in [25]. Briefly, BSM was dissolved overnight at 4 °C using a nutating mixer in 10 mM Na-acetate, 1 mM EDTA, pH 5.0 buffer with a final concentration of 10 mg/mL. The solution was clarified through sterile filtration (5 µm hydrophilic polyethersulfone sterile filter; Pall Corporation,
Cornwall, U.K.) and subsequently fractionated according to charge on a high load 16/26 Q Sepharose high performance anion exchange column (GE Healthcare Life Sciences, Uppsala, Sweden) installed on an Äkta Avant Chromatograph (GE Healthcare, Uppsala, Sweden). Proteins bound on the column were fractionated with a multi-step gradient of high salt elution buffer (10 mM Na-acetate, 1 mM EDTA, 1.2 M NaCl, pH 5.0). Protein containing fractions were analyzed using SDS-PAGE coomassie brilliant blue staining to visualize proteins. BSM fractions were pooled, dialyzed against milliQ grade water at a ratio of 1:400 and subsequently freeze-dried. All steps were performed at 4°C to minimize potential proteolytic degradation.

**Proteolytic digestion of BSM**

For trypsin proteolysis, BSM was dissolved in 50 mM NH₄HCO₃ to a final concentration of 10 mg/mL with a sample size of 100 µL. Any disulfide linkages present were reduced by addition of 5 µL DTT (200 mM DTT in 100 mM NH₄HCO₃) and left a room temperature for 1 hour. Alkylation was stopped by adding 4 µL iodoacetamide (1M iodoacetamide in 100 mM NH₄HCO₃) and incubating at room temperature for 1 hour. The alkylation reaction was quenched by adding an additional 20 µL DTT solution. 40 µg of trypsin was added to the solution to a final ratio 1:25 and incubated at 30 °C overnight. After incubation, 831 µL PBS buffer was added to the reaction for a final BSM concentration of 1 mg/mL.

BSM was dissolved in 5% formic acid to a final concentration of 10 mg/mL for pepsin proteolysis. Disulfide linkages were reduced identical to the procedure described above. 50 µg of pepsin in 5% formic acid was added to a final ratio of 1:20 and the samples were incubated overnight at 30 °C. 831 µL PBS buffer was added to the sample for a final BSM concentration of 1 mg/mL. To verify that proteolytic cleavage of the peptide had occurred, 100 µL of either trypsin- or pepsin digested BSM was analyzed by SDS-PAGE using to staining methods; coomassie brilliant blue
(CBB) was employed to visualize peptide fragments while periodic acid/Schiff (PAS) staining was used to detect the presence of glycosylated peptides.

**Matrix assisted Laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS)**

Visible bands on the CBB stained gel of peptidase treated BSM were excised with a scalpel and transferred to Eppendorf tubes where they were washed with 40% EtOH until colorless. The EtOH was removed and 100% acetonitrile was added and incubated until the gel fragments were dehydrated. The peptides were extracted from the gel fragments by removing the acetonitrile and adding 25 mM NH₄HCO₃ and incubating overnight at 37 °C. A supernatant aliquot (1 µL) was applied to the anchor chip target (Bruker-Daltonics, Bremen, Germany), covered by 1 µL matrix solution (0.5 µg/µL of α-cyano-4-hydroxycinnamic acid (HCHA), 90% acetonitrile, 0.1% TFA) and washed by addition of 2 µL TFA. Excess was removed after 30 s. MS spectra were obtained using an Ultraflex II MALDI-TOF MS mass spectrometer (Bruker-Daltonics) in auto-mode using flex control v3.0 (Bruker-Daltonics) and processed by flex analysis v3.0 (Bruker-Daltonics). Peptide mass maps were acquired in reflectron mode with 500 laser shots per spectrum. Spectra were externally calibrated using a tryptic digest of β-lactoglobulin (5 pmol/µL). The MS spectra were searched against the NCBI nr database for mammals (database nr. 56546546) using the MASCOT 2.0 software (http://www.matrixscience.com) integrated together with BioTools v3.1 (Bruker-Daltonics). Filtering of spectra was carried out for peaks corresponding to known fragments from keratin and trypsin autocatalysis.

**Size exclusion (SEC) chromatography**

BSM samples were dissolved to a final concentration of 3 mg/mL and clarified by filtration (5 µm hydrophilic polyethersulfone sterile filter; Pall Corporation, Cornwall, U.K.) to remove aggregates.
6 mg of sample was loaded on a HiPrep 26/60 Sephacryl S1000 Superfine gel filtration column (GE Healthcare Life Sciences, Uppsala, Sweden) installed on an Äkta Avant chromatograph (GE Healthcare, Uppsala, Sweden). The column volume was 318 mL and the samples were eluted with PBS buffer at flow rate of 0.5 mL per minute. Chromatograms were recorded according to absorbance at 214 nm which corresponds to peptide linkage emission.

**Circular Dichroism (CD) Spectroscopy**

Circular dichroism (CD) spectra of enzymatically treated BSM were acquired by employing a rectangular quartz cuvette with 0.5 mm path length (Hellma GmbH & Co. KG, Müllheim, Germany) using Chirascan spectrophotometer (Applied Photophysics Ltd., Surrey, UK). The concentration of the enzymatically treated BSM in the cuvette was 1 mg/mL. Spectra of the samples were recorded in the far-UV range from 260 to 195 nm with a step size of 1 nm, a bandwidth of 0.5 nm and a time-per-point value of 1.5 s. One measurement was obtained from averaging three traces. All samples were measured thrice and then averaged, and the background from buffer was subtracted.

**Dynamic Light Scattering (DLS)**

Enzymatically treated BSM samples (1 mg/mL) were dialyzed using a 1 mL Float-a-lyzer dialysis membrane with a 100 kDa cutoff against PBS buffer. Samples were measured using a Malvern Zetasizer Nano ZS two angle particle and molecular size analyzer (Malvern Instruments, Worcestershire, UK). The non-invasive back scatter (NIBS, measurement angle 173°) was used to achieve highest sensitivity. The temperature was set at 25 °C and the He-Ne laser light source to a wavelength of 633 nm. Disposable cuvettes (PMMA, Plastibrand) were employed for DLS measurements. Each sample was measured in triplicates. The Malvern Zetasizer software (Version
7.02) was used to analyze the obtained data and intensity distribution of the hydrodynamic diameter (Dₜ) was chosen as the display mode.

**Optical Waveguide Lightmode Spectroscopy (OWLS)**

OWLS is based on grating-assisted in-coupling of a He-Ne laser into a planar waveguide coating (200-nm thick Si₀.₂₅Ti₀.₇₅O₂ waveguide layer on 1 mm thick AF 45 glass (Microvacuum Ltd, Budapest, Hungary). Adsorption of biomolecules from bulk liquid to the interfacing solid surface is measured by monitoring changes in the refractive index close to the solid-liquid interface. This method is highly sensitive out to a distance of ~200 nm from the surface of the waveguide. Experiments were carried out using an OWLS 210 Label-free Biosensor system (Microvacuum Ltd, Budapest, Hungary).

For consistency between surface adsorption measurements and the tribology measurements described below, the waveguides that were used in this work were coated with a layer of poly(dimethylsiloxane) (PDMS). The waveguides were spin-coated at 2,500 rpm for 15 s firstly with an ultrathin layer (ca. 24.3±3.1 nm, determined by scratch test using atomic force microscopy in tapping mode) of polystyrene (Sigma Aldrich, Brøndby, Denmark), dissolved in HPLC grade toluene at 6 mg/mL. A subsequent ultrathin layer of PDMS (ca. 16.4±0.17 nm) was applied [26].

The base and curing agent of a commercial silicone elastomer (Sylgard 184 elastomer kit, Dow Corning, Midland, MI) were dissolved in hexane at a ratio of 10:3 (final concentration, 0.5 % w/w). The solution was spin-coated onto the waveguides at 2,000 rpm for 25 s, and cured in an oven at 70 °C overnight.

The PDMS-coated waveguide was exposed to PBS buffer to obtain a stable baseline using a programmable syringe pump (Model 1000-NE, New Era Pump Systems, Inc., NY) to pump buffer solutions through the flow-cell across the OWLS waveguide surface. 100 µL of dialyzed enzymatically treated BSM sample (1 mg/mL) was then injected via a loading loop. Upon
observing surface adsorption, the pump was stopped so that the BSM molecules could adsorb onto surface under static conditions. After 10 min, the flow cell was rinsed with PBS buffer by restarting pumping. The adsorbed mass density data were calculated according to de Feijter’s equations [27]. The experiment was repeated two or three times for each type of enzymatically treated BSM. A refractive index increment \((\text{dn/dc})\) value of 0.150 cm\(^3\)/g for BSM was used for the calculation of the adsorption masses [19].

**Pin-on-Disc (PoD) Tribometry**

Macroscale tribological properties of enzymatically treated BSMS were characterized with a pin-on-disc tribometer (CSM, Peseux, Switzerland). In this approach, a loaded pin was allowed to form a contact with a disc and the sliding friction forces between them were measured at controlled rotation speeds of the disc. The load on the pin was applied with a dead weight (1 N) while sliding speed was controlled by rotating the disc with a motor. Friction forces generated during sliding contacts were monitored by a strain gauge on the arm holding the pin. The friction forces data obtained in this study were measured as a function of number of rotations (20) over a fixed track (radius: 5 mm) while varying the speed of rotation. An average coefficient of friction, defined as friction force/load, for each speed could then be plotted.

The PDMS tribopair used in this study was prepared with the Sylgard 184 elastomer kit described above (Dow Corning, Midland, MI, USA). The base fluid and cross linker were thoroughly mixed at a ratio of 10:1. Air trapped in the mixture was removed by applying a gentle vacuum. Discs were cast in a machined aluminum plate mold with flat wells with the dimensions of 30 mm in diameter and 5 mm in depth. The surface exposed to air in the course of curing was used for tribological measurements. The hemispherical pins (6 mm in diameter) were cast in a 96 microwell plate (NUNCLON Delta Surface, Roskilde, Denmark). The PDMS mixtures were subsequently cured at 70 °C overnight.
Results and Discussion

Proteolytic digestion of BSM

BSM was digested with two different peptidases to investigate whether any difference would be observed according to cleavage sites. Trypsin specifically cleaves the peptide on the C-terminal side of either lysine or arginine amino acid residues [28]. Pepsin, on the other hand, exhibits preferential cleavage for hydrophobic, preferably aromatic, residues [28]. Figure 2 Shows the SDS-PAGE data obtained after peptidase digestion. CBB staining was employed to visualize the position of the protein part of the macromolecule in the gel, while PAS staining selectively dyes the oligosaccharides. BSM has previously been reported to be visible in the high molecular weight range above 200 kDa which was also the case here using both CBB and PAS staining [25]. Both trypsin-digested BSM (Trp-BSM) and pepsin-digested BSM (Pep-BSM) displayed fragments in the low molecular range on the CBB stained gel (Trp and Pep, respectively, in Figure 2) indicating that proteolytic degradation of the protein had occurred. MALDI-TOF MS analysis identified the excised bands as originating from BSM (data not shown). The fragments observed from Trp-BSM are found in distinct bands at approximately 7 kDa, ~4 kDa and ~3kDa. This corresponds approximately to the fragment sizes that were expected by a theoretical digestion of an N-terminal partial sequence of 1589 amino acids residues where the largest fragment had a mass of 7152 Da when allowing for one missed cleavage [29]. The theoretical digestions for both trypsin and pepsin can be found in the Supplementary Information. For Pep-BSM, a distinct band at ~36 kDa was identified to be pepsin on the CBB stained gel. Additionally, a smear can be seen in Figure 2 with three distinct bands at ~21 kDa, ~15 kDa and ~5 kDa. The largest peptide in the theoretical digest of the partial sequence had a mass of 12783 Da, thus not corresponding to what is seen on the gel. A possible explanation is that the large peptides identified originate either from several missed
cleavages or from the C-terminal part of the protein. PAS staining showed a dense smear for BSM above 200 kDa on the gel (Figure 2). For Trp-BSM and Pep-BSM, the smear migrated to a lower molecular weight position than BSM. However, the peptides in the low molecular range of the gel have not been stained indicating that those peptides are non-glycosylated. These fragments therefore most likely originated from the terminal regions of the protein. Furthermore, as expected the oligosaccharides either shield any digestion sites that are present in the central mucin domains or there are simply no sites in the sequence.

![Figure 2 SDS-PAGE gels stained with CBB (left) and PAS (right). Intact BSM is indicated by a “B”, Trypsin digested BSM by “Trp” and pepsin digested BSM by “Pep”.

Size of protease digested BSMs

With protease digestion, the size of BSM should be reduced compared to that of untreated BSM. SEC and DLS were employed to monitor any changes to the size of the molecules after proteolytic
treatment, and the results are presented in Figure 3 and 4, respectively. In SEC, a porous gel bead material is employed in the column and the size of proteins determines the elution time. Porous nature of the material allows for smaller proteins to enter the gel beads where they have to traverse the gel matrix inside the beads and thus their flow through the material is effectively retarded. Larger proteins that do not enter the beads have a shorter path through the column material as they move around the gel beads. Elution from a SEC column therefore goes from larger proteins first, followed by smaller species according to their size. Figure 3 shows the chromatograms obtained by loading 6 mg of sample onto the column. Intact BSM (Figure 3, black) was used as a standard to monitor changes in the digested samples. The intact BSM eluted as a single peak between 150 mL to 250 mL and over a total volume of approximately 100 mL. Furthermore, DLS (Figure 4) showed a single peak distribution of intact BSM with an average hydrodynamic diameter of 50 nm. The chromatogram recorded for Trp-BSM in Figure 3 shows two distinct peaks that have eluted at 275 mL and 350 mL downstream of the intact BSM peak, indicating that they are smaller species. Thus, the size of BSM has been reduced which is in accordance with what was expected from proteolytic degradation. The DLS data, however, shows multiple peaks for Trp-BSM where the largest has a hydrodynamic radius of 295 nm and the smaller is identical to that of intact BSM (50 nm) (Figure 4). The presence of the smaller peak that is identical to BSM may indicate incomplete proteolytic degradation of the sample. The peak at 295 nm is most likely a product of aggregation between digested BSMs. Although as the sample is retarded in the SEC column material in Figure 3, the interaction between degraded Trp-BSMs may be very weak, leading to their elution being later than that of BSM.
Figure 3 SEC of BSM (black), Trp-BSM (red) and Pep-BSM (blue). 6 mg of sample was loaded onto the column and the elution volume was monitored by absorbance at 214 nm.

The chromatogram for Pep-BSM shows three peaks that elute at 75 mL, 118 mL and 155 mL indicating that the size of Pep-BSM has increased after proteolytic degradation. This correlates with the DLS data in Figure 4 where the average hydrodynamic size of the Pep-BSM is 531 nm. This may indicate that Pep-BSM form strongly associating aggregates. The restriction sites that are present in the terminal regions of the protein may possibly cleave the peptide in a way that exposes long sequences of internal hydrophobic amino acid residues thus making them more solution exposed. Hydrophobic regions tend to interact to shield themselves from the solvent and thus intermolecular interactions between BSMs are highly likely to occur. A small additional peak is present in Figure 3 that has eluted at 260 mL. This peak could possibly correspond to non-aggregated Pep-BSM as it has eluted after the non-treated BSM. At the same time the smaller peak with a hydrodynamic size of 15 nm in the DLS indicates that non-aggregated Pep-BSMs (or a sizeable peptide that was not removed by dialysis) may also be present in solution.
Changes in the secondary structure of BSM due to proteolytic degradation

BSM has been shown to display a spectrum that corresponds to a random coil structure as there are no detectable features of α-helices or β-sheets present in the spectrum (Figure 5) [30-33]. However, the structural features are also reminiscent of a poly Pro (II) helical structure though the position of the maxima and minima are shifted slightly compared to a pure poly Pro (II) helix [33]. The spectrum recorded for BSM in this study is similar to those reported previously for BSM with a local maximum at ca. 220 nm and a minimum at ca. 203 nm [25, 34]. Furthermore, as previously reported, no tertiary structural features were recorded for BSM [25]. Figure 5 shows the spectra recorded for intact BSM and protease treated BSMs.
The Trp-BSM and Pep-BSM samples were also analyzed by far-UV CD spectroscopy. It was found that the peptides present in the samples after proteolytic degradation interfered with the signal (data not shown), and thus the samples were subsequently dialyzed. The spectra recorded for dialyzed Trp-BSM and Pep-BSM have a similar positive maximum at ca. 220 nm (Figure 5). The minima for both samples, on the other hand, have shifted slightly to ca. 200 nm and the intensity of the spectra is slightly diminished compared to that of intact BSM. It is not surprising that the overall secondary structure of Trp-BSM and Pep-BSM have not changed drastically as the vast majority of BSM is made up of the glycosylated central domains. As the oligosaccharides most likely shield the peptide from the proteases, the majority of the protein would not have been cleaved due to physical blocking of the cleavage site. However, as the samples were dialyzed prior to measurement, the reduction in signal intensity could arise from a reduction in protein concentration i.e. the peptide fragments from the terminal domains were removed from the solution. The slight shift to a shorter wavelength could originate from further disordering in the structure of BSM. The terminal domains mucins are known to contain many structural motifs such as vWF-domains and cysD domains [7, 8].
The proteolytic degradation of these structures and subsequent removal of the peptides could very well be the cause of the shift in the position of the local minimum.

**Adsorption and tribological behavior of BSM after proteolytic digestion**

Mucins are usually described as having a “dumbbell” like structure with the hydrophobic globular terminal domains separated by the heavily glycosylated central domains as was depicted in Figure 1. OWLS was employed to monitor the adsorption of the protease digested BSMs onto a hydrophobic PDMS surface. The treated samples were dialyzed prior to measurement, so as to avoid “noise” from the peptide fragments present in solution. Intact BSM was also measured and the adsorbed mass was determined to be 43.05 ± 0.05 ng/cm². In comparison, Trp-BSM adsorbed in a substantially lower mass with only 12.79 ± 0.64 ng/cm². Pep-BSM showed inverse behavior as the adsorbed mass was found to be slightly higher than that of whole BSM at 53.30 ± 0.09 ng/cm². As both of the proteases most likely only cleave the exposed peptide in the terminal domains of the protein, the explanation may lie in the frequency and position of the cleavage sites present in the sequence. The theoretical digestions (in the Supplementary Information) of the partial sequence of the N-terminal part of the protein show that many more cleavage products are generated by trypsin. With the higher frequency of cleavage sites for trypsin there is a significant chance that a cleavage site is located in close proximity to the central domains in the primary sequence. Thus the remaining non-glycosylated part of the protein would be severely reduced, causing the overall adsorption to drop in the flow cell as the “anchoring” domains of the protein are diminished. This clearly confirms that adsorption of BSM onto a hydrophobic surface such as PDMS is achieved via the interaction of the terminal domains with the hydrophobic substrate.
As previously mentioned in the discussion of the SEC and DLS results, Pep-BSM appears to aggregate in solution. The lower frequency of unique proteolytic cleavage sites in the primary structure of the terminal domains of BSM may explain the increase in the adsorbed mass for Pep-BSM. Unlike Trp-BSM, Pep-BSM most likely retains a substantial part of its terminal domains after proteolytic cleavage. The protease treatment may have exposed hydrophobic sequences in the primary structure that were previously buried within the structure of the globular terminal domains. In solution the hydrophobic parts of the individual BSMs would seek to shield themselves from the solution and aggregates may therefore form. The increase in the adsorbed mass could correspond to the formation of stable aggregates that have adsorbed onto the surface. Aggregates are usually associated with a lowering of the total adsorbed mass in OWLS measurements of BSM due to intermolecular repulsion and taking up a large space on the surface [34]. However, as BSM is already a highly negatively charged molecule at neutral pH, the intermolecular repulsion is already high between BSMs. Thus the adsorption of the aggregates only slightly increased the adsorbed mass compared to whole BSM (Figure 6).
Changes that occurred in the tribological properties of BSM were investigated using a PoD tribometer. Figure 7 shows the results for PBS buffer, intact BSM and the protease digested BSMs. Measurements were made from highest speed to lowest.

![Graph showing coefficient of friction against speed for PBS buffer (grey), BSM (black), Trp-BSM (red), and Pep-BSM (blue).](image)

Figure 7 PoD results for PBS buffer (grey), BSM (black), Trp-BSM (red) and Pep-BSM with the coefficient of friction plotted against the speed.

As a reference, the friction generated between bare PDMS surfaces in PBS buffer was recorded (Figure 7), which was found to be in the range of 1 – 1.1 within the measured speed range. Intact BSM shows a consistently low coefficient of friction, namely in the range of 0.02, over the entire speed range, indicating a maintenance of a lubricious BSM film under the tribostress; even if initially adsorbed BSMs are rubbed away from the tribostress, the lubricating films are rapidly regenerated from the rapid adsorption of molecules from the bulk solution. Trp-BSM (Figure 7, red) shows fairly effective lubricating properties in the high speed regime, but loses the lubricity rapidly below 50 mm/s. It is most likely a consequence the low mass adsorption that was observed in Figure 6. The low coverage of the PDMS surfaces with Trp-BSM may induce partial direct asperity contacts between bare PDMS surfaces and thus cause higher interfacial friction forces. Similar
results were obtained for Pep-BSM (Figure 7, blue); the lubricating effect of Pep-BSM is comparable to intact BSM only in the high speed regime due to facile entrainment into the tribological interface, but is clearly inferior in the low-speed regime. As the adsorbed mass of Pep-BSM onto PDMS is slightly higher than that of intact BSM (Figure 6), low surface coverage as with the case of Trp-BSM, cannot account for its behavior. Instead, newly formed aggregates of BSM after pepsin digestion may not be strong and stable enough to withstand the tribological stress, even though they are sufficient to adsorb onto PDMS surface. For example, the severely “stunted” remains of the globular terminal domains in Trp-BSM may be shielded by even larger glycosylated domains, thus lessening the ability to retain the BSM molecules on the PDMS surface under tribostress. Nevertheless, the friction coefficients for Pep-BSM are still lower than those of Trp-BSM, and the onset of degrading lubricity starts at somewhat lower speed than Trp-BSM. This observation supports that the origin of the decreased lubricating properties of Trp-BSM and Pep-BSM is somewhat different: Trp-BSM due to poor adsorption (near complete removal of terminal regions) vs Pep-BSM due to weak adsorption (aggregation due to exposed hydrophobic patches).

Overall, the adsorption- and PoD data suggest that the terminal domains of BSM are vital for efficient adsorption and aqueous lubrication for hydrophobic interfaces, and intact BSM has an optimal structure/composition for it.

**Conclusion**

In this work we addressed the effect of proteolytic degradation by two distinct proteases on the structure and conformation of BSM in bulk solution and its adsorption and tribological properties at an elastomeric hydrophobic surface of PDMS. The proteolytic digestions of BSM using the proteases trypsin and pepsin were verified using SDS-PAGE. The peptide specific stain CBB showed that proteolytic cleavage had occurred while the carbohydrate specific PAS stain confirmed that none of the peptides contained carbohydrate modifications. Size exclusion chromatography
showed that protease-cleaved BSMs had been changed in size. Trp-BSM eluded after intact BSM in two distinct peaks indicating that the size of the molecule had been reduced. Pep-BSM, on the other hand, had increased in size and eluded from the column before the whole BSM in three distinct peaks. This was most likely due to formation of aggregates as a small peak was found to elute after intact BSM. The small peak may correspond to either non-aggregated Pep-BSM or a large peptide product. DLS was employed to verify the changes in the hydrodynamic size of the protease digested BSMs. Trp-BSM exhibited two peaks, one corresponding to the size of intact BSM and a peak with a larger hydrodynamic size. This indicated firstly that the sample may not have been fully digested but also that Trp-BSM in solution starts to form aggregates. Pep-BSM displayed a peak that was 10 times larger than the hydrodynamic size of intact BSM, indicating that an increase in aggregation of BSM takes place after pepsin cleavage. This correlates with the data from SEC. A peak was also recorded at 15 nm that could correspond to either single Pep-BSMs or possibly a large peptide.

Changes in the secondary structure of BSM were monitored using CD spectroscopy. Both Trp-BSM and Pep-BSM were found to have slightly shifted minima to ca. 200 nm from ca. 220 nm when compared to intact BSM. This could be indicative of a slight increase in disordering of the structure from protease treatment. The general features of the spectra did not change and generally the random coil secondary structure was maintained after protease treatment. This further validates the central tandem repeat domains of BSM were not degraded by protease treatment. Adsorption of BSM onto surfaces is necessary for effective aqueous boundary lubrication. OWLS was used to monitor the adsorption behavior of the protease-digested BSMs. Trp-BSM showed a significant decrease in its adsorption, indicating that the overall adsorption properties were quite diminished compared to intact BSM. This may be due to the terminal domains being severely stunted. Pep-BSM on the other hand displayed an increase in the adsorbed mass compared to whole BSM. This is in agreement with the findings from DLS and SEC measurements and is an indication that
aggregation occurs between the Pep-BSMs. The lubricating properties of BSM have been well established and the measurements in this study are in agreement with previous findings [35]. The lubrication properties of both Trp-BSM and Pep-BSM were found to degrade rapidly upon reaching slower speeds, yet the former being somewhat more severely than the latter. While both are related to the reduced surface adsorption of the protease-treated BSMs, the more “stunted” remains of the globular domains in Trp-BSM result in weak surface binding and subsequent poor surface coverage. The poor performance of Pep-BSM probably originates from the reduction in the size of the globular terminal domains and the formation of large aggregates that are easily dissociated from the surface during tribostress.

The data presented in this paper validates that the hydrophobic terminal domains of BSM are pivotal for effective adsorption and aqueous lubrication on soft hydrophobic surfaces.

References


Supplementary information

Theoretical digestion of the partial N-terminal sequence of BSM

The partial N-terminal sequence of BSM determined by Jiang et al. was copied into peptide mass determination program at expasy.org.

Trypsin digestion of partial BSM sequence

Parameters: Maximum number of missed cleavages = 1. Cysteines have been treated with iodoacetamide. Fragments below 500 kDa omitted. Monoisotopic masses have been used.

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Pepsin digestion of partial BSM sequence

Parameters: Maximum number of missed cleavages = 1. Cysteines have been treated with iodoacetamide. Fragments below 500 kDa omitted. Monoisotopic masses have been used.

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| 5103.5676 | 2980.4453 | 1834.0141 | 862.4451 |  |
| 4905.4500 | 2974.4447 | 1832.9236 | 776.4148 |  |
| 4853.4219 | 2952.5120 | 1806.7309 | 767.3682 |  |
| 4848.2227 | 2893.4385 | 1920.7738 | 753.3413 |  |
| 5190.3515 | 2885.4082 | 1767.9083 | 745.3726 |  |
Conclusions

In this work we established a simple one column anion exchange chromatography protocol for the purification of commercial BSM that recovered a high yield of purified BSM from the initial mass. The importance of standardizing the BSM samples arose from the reports of significant batch-to-batch variation in the protein content of commercial BSM. The ability to standardize the samples for comparative purposes was therefore stressed. BSM recovered from this purification method gave homogenous samples with a size distribution in the range of 40 – 48 nm as measured by DLS. Other non-mucin biomolecules were identified in the commercial BSM. The most abundant “contaminant” was BSA.

Commercial mucins have been used in many studies concerning their physicochemical properties, such as their lubricant ability between sliding surfaces. Many of these studies employed the mucins either “as received” (arBSM) or after mild purification by dialysis (dBSM). We compared the tribological properties of arBSM, dBSM and purified BSM (aeBSM) at the macro- and nanoscale. At the macroscale, inferior lubricity was observed for arBSM due to the amount of lighter molecules such as BSA adsorbing faster to- and thus dominating the surface compared to the larger BSM molecules. At the nanoscale, it was found that for all cases, the friction was higher than that of the buffer due to the tip plowing through the mucinous layer. Friction was found to increase with the content of impurities due to an increase in the viscosity of the layer.

BSA was determined to be the most abundant non-mucin protein present in commercial BSM. We investigated the interaction between BSM and BSA using spectroscopic approaches. A ratio of 10:1 between BSM and BSA was used as the content of BSA in commercial BSM was determined to be ~9% by weight in a previous study. Well characterized tryptophan fluorescence emission was employed to monitor changes induced either by protein-protein association or changes in the tertiary conformation of BSA. When freshly mixed, a red shift along with an intensity drop in the emission spectrum after incubation, indicate that the surface tryptophan in BSA has become more solution exposed. This could be due to associating with the terminal domains of BSM. When BSM is incubated extensively prior to mixing with BSA, a blue shift accompanied by a 50% drop in emission intensity was recorded. This indicates that the surface tryptophan residue in BSA had become more shielded from solution. The extensive incubation of BSM may cause either intra- or intermolecular associations in BSM that shield the previous interaction sites, thus, forcing BSA to
possibly form non-specific interactions with the oligosaccharides of the central domains of BSM instead. No extensive complex formation was observed by DLS in this study.

We showed that BSM is highly thermostable when heating to temperatures beyond the physiological range of most proteins. The secondary structure of the protein was shown to undergo appreciable changes when heating from 5 to 85 °C. However, the changes were fully reversible, and, even extended heating was also shown to not impact the secondary structure of BSM irreversibly. DLS showed that BSM did start to aggregate with extended heat treatment. This was also reflected in the surface adsorption mass that decreased slightly due to reduction in packing efficiency. The tribological properties of BSM were found to deteriorate at elevated temperatures, but were retained upon cooling. The thermostability of BSM most likely arises from the largely random coil structure of the molecule.

The properties of mucins from two different origins, namely BSM and PGM, were investigated under neutral- and acidic conditions. It was shown that no structural changes occurred within the mucins under either condition. However, differences between the mucins were recorded. While the far-UV spectra of PGM appears to display a fully random coil structure, BSM showed some structural similarity to that of a poly pro (II) helical conformation. This may be due to the differences in the oligosaccharides of the central domains. No tertiary structure was observed for either mucin. Emerging aggregation was recorded for BSM under acidic conditions. This was also reflected in the surface adsorption properties where the surface mass was doubled. No changes were recorded for PGM. QCM-D showed a less dense film formation for PGM on the surface when compared to BSM. This was reflected in the tribological properties where both BSM and PGM displayed increased lubricity under acidic conditions. However, the lubricity of PGM failed in the low speed regime. This may be related to poor film stability on the surface.

Finally, it was shown that the globular, hydrophobic terminal domains of BSM are responsible for effective surface adsorption to, and subsequent lubrication of, a hydrophobic surface. Two proteases, tryptophan and pepsin, that have distinctively different proteolytic recognition sites, were used to treat intact BSM. SDS-PAGE analysis by different staining techniques showed that the central domains were not degraded by the proteases and, thus, the terminal domains only were targeted. This was further confirmed by CD spectroscopy as their spectra displayed random coil conformations. SEC and DLS analysis showed that the size of the protease treated BSM increased, which indicates that aggregation of the protein had occurred. Surface adsorption studies exhibited
an increase in mass uptake for pepsin treated BSM corresponding to the adsorption of large aggregates on the surface. Trypsin treated BSM, on the other hand, displayed a decrease in mass adsorption that is most likely a result of poor anchoring onto the surface. The lubricating properties of the protease degraded BSMs were found to be severely reduced, thus, confirming that the hydrophobic globular terminal domains are responsible for effective adsorption onto hydrophobic surfaces.

**Future perspectives**

Commercially available mucins are an excellent starting point for studying the surface properties afforded by the molecules. However they do have one un-ignorable drawback in that no information about the method of isolation of the proteins is actually accessible to the customer. Their isolation may very well include steps where the material has been treated in a way that causes irreversible changes to the protein, such as denaturation. It is therefore imperative to establish a method for obtaining purified mucins directly from tissue samples. This would remove any doubt about the integrity of the samples.

Secreted mucins generally have the same conformation with a similar distribution of structural motifs in their globular terminal domains such as the vWF-like domains. The main diversity displayed by mucins is found in the oligosaccharide posttranslational modifications. A logical step from the work presented in this thesis is to explore the impact of modifying the composition of the oligosaccharides. PGM and BSM were shown to display different properties according to pH even though the vast majority of the apoprotein is made up of the PTS repeat central domains. Selective deglycosylation of carbohydrate moieties in the oligosaccharides attached to the central domains may divulge important information about the lubricating properties of the mucins.

Although mucins serve as an excellent model system for biocompatible lubricants, there are still many factors that need to be taken into consideration. Particularly the host immune response to mucin coated surfaces should be prioritized in future studies where mucin coatings are considered. The apparent complications that could arise from a host immune response are of great concern. Using a human mucin-producing cell line may therefore be a more viable route towards an applicative mucin coating in the future. Using human mucins may also potentially reduce
complications in regards to the recognition by the immune system of mucins as “other”. The extensive knowledge about not only the structure of mucins, but also about their surface adsorption- and tribological properties may serve as inspiration towards the development of artificial surface coating materials that are biocompatible. Synthetic polymers are an excellent base material that could potentially be utilized. However, features of the mucin structure may prove to be very complex to mimic, particularly the complexity of the posttranslational oligosaccharides may pose a challenge.
References


Appendix 1: Adsorption and nanowear properties of bovine submaxillary mucin films on solid surfaces: Influence of solution pH and substrate hydrophobicity

In this study, we were interested in using an AFM nanowear technique to characterize the nanoscale mechanical stability of bovine submaxillary mucin (BSM) films formed at solid–liquid interfaces. Particular attention was paid to the mechanical stability in variation of solution pH and substrate hydrophobicity.

My contributions to the work was supplying purified BSM, adsorption measurements by OWLS and writing the experimental parts for the respective techniques.
Adsorption and nanowear properties of bovine submaxillary mucin films on solid surfaces: Influence of solution pH and substrate hydrophobicity

Javier Sotres, Jan Busk Madsen, Thomas Arnebrant, Seunghwan Lee

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The adsorption and mechanical stability of bovine submaxillary mucins (BSM) films at solid–liquid interfaces were studied with respect to both substrate hydrophobicity and solution pH. Dynamic light scattering revealed a single peak distribution in neutral aqueous solution (pH 7.4) and a small fraction with enhanced aggregation was observed in acidic solution (pH 3.8). Both substrate hydrophobicity and solution pH were found to affect the spontaneous adsorption of BSM onto solid surfaces; BSM adsorbed more onto hydrophobic surfaces than hydrophilic ones, and adsorbed more at pH 3.8 than at pH 7.4. Thus, the highest “dry” adsorbed mass was observed for hydrophilic surfaces in pH 3.8 solution. However, a highest “wet” adsorbed mass, i.e. which includes the solvent coupled to the film, was observed for hydrophobic surfaces at pH 7.4. The mechanical stability of the films was studied at the nanoscale with an atomic force microscope operated in the friction force spectroscopy mode. Results revealed that BSM films formed on hydrophobic substrates were stronger than those formed on hydrophilic ones. Moreover, the film stability also depended on the ambient pH and stronger films were formed at acidic conditions, i.e. close to the BSM isoelectric point.

1. Introduction

Mucins represent the major macromolecule comprising the mucus gels or glyocalyx on the epithelial cell membranes. Biological functions of mucus gels are known to range from the protection of underlying tissues against external insult, mechanical stress, and pathogens [1,2] to cell signal transduction [3]. Inspired from their protective biological functions, mucins started to attract interests as coating materials for implants and biomedical devices [4–8]. Previous studies on this topic have shown that mucin coatings on surfaces display favorable immune responses [6], suppression of bacterial adhesion [4,7], and controlled adhesion of various biomolecules [8].

Enormous efforts are devoted nowadays within various disciplines to study biological molecules at surfaces [9–11]. In this study, we are particularly interested in mucins from oral fluids and their interaction with surfaces. Mucins are a key component of saliva, the biofluid secreted into the oral cavity that is mainly composed of water (up to 99%) and a wide variety of electrolytes, lipids, and proteins [12]. A striking characteristic of saliva is that it adsorbs immediately upon contact with any type of surfaces [13], forming a protective layer, i.e. the pellicle. Interestingly, mucins are present not only in bulk saliva but have also been reported to be among the most abundant components of the pellicle [14]. One of the functions of adsorbed salivary films is the protection of oral surfaces against acidic corrosion [15] and mechanical abrasion [16]. The mechanisms underlying this protective function are not yet fully understood. It is, however, well accepted that one of these mechanisms is efficient boundary lubrication [17]. In parallel, mucin films on surfaces are also known to exhibit excellent boundary lubricating properties [18]. As with most other lubricants, it has been specifically shown that the glycosylated components, most probably mucins, are mainly responsible for the lubricative properties of the pellicle [19]. The critical role of mucins in oral lubrication has rendered these molecules to be included as a key component of many salivary substitutes [20]. However, the stability of mucin films and the relating protection against mechanical and chemical attacks have not been studied to date. Therefore, it would be of interest to investigate these aspects of mucin films and compare results with those recently obtained for salivary films [21–24] in order to elucidate the role of mucins in protecting oral surfaces.

Atomic force microscopy (AFM) has emerged as a powerful technique for studying the mechanical stability of adsorbed thin
films at the nanoscale. Indeed, this is the main scale where studies on the mechanical stability of protein films should focus as both the size of proteins and the thickness of the films they form typically lie in the nanometer range. One of the most common approaches to study the mechanical stability of adsorbed thin films by means of AFM is to generate wear on i.e. to scratch, them by scanning their surfaces while continuously varying the load. Wear resistance of the film is subsequently characterized either by monitoring the friction force between tip and sample [25] or by acquiring the topography of the scratched area after the experiment [26].

Recently, an improved methodology for studying nanoscale wear of soft adsorbed films was introduced. It consists of simultaneously monitoring the friction force and the surface topography during the scratch of the film [27]. This allows for a clearer identification and more precise characterization of critical wear events such as the rupture and removal of the films along with the forces exerted during these events.

In this study, we have used this approach to characterize the nanoscale mechanical stability of bovine submaxillary mucin (BSM) films formed at solid–liquid interfaces. A particular attention was paid to the mechanical stability in variation of solution pH and substrate hydrophobicity. Specifically, BSM films formed both on bare (hydrophilic) and methylated (hydrophobic) silica surfaces were studied in phosphate buffered saline (PBS) solutions with pHs 7.4 and 3.8. In order to help interpreting results, other surface parameters of the BSM films as well as of the BSM molecules in the bulk solution have been characterized. For surface parameters, adsorbed “dry” mass was characterized by means of optical waveguide lightmode spectroscopy (OWLS) whereas hydrated mass and surface rheological properties of the films were characterized by means of quartz crystal microbalance with dissipation (QCM-D). For bulk parameters, the aggregation state of the molecules in the bulk solution was characterized by means of dynamic light scattering (DLS). Results show that the mechanical stability of the films correlates with their dry mass to some extent, and both quantities increase significantly with the hydrophobicity of the substrate. Additionally, this stability also increases at acidic condition where pH approaches the isoelectric point of BSM as well as the employed substrates (SiO₂), implying that the aggregation of BSM molecules is more enhanced at the solid/liquid interface than in bulk solution.

2. Materials and methods

2.1. Chemicals and buffer solutions

All chemicals were of analytical grade or higher purity, unless otherwise stated. Water was treated by a purifying unit (ELGA UHQ PS, Elga Ltd. UK or Merck Millipore, Billerica, MA) until a resistivity higher than 18 MΩ cm was attained.

2.2. BSM and the purification

BSM was obtained from a commercial resource (M3895-1 G, Type I-S, Sigma–Aldrich, St. Louis, MO). Since commercially available mucins are known to contain non-mucin impurities, BSM was further purified by means of anion exchange chromatography [5]. BSM received from the manufacturer (“ar-BSM”) was dissolved in Na-acetate buffer (10 mM Na-acetate, 1 mM EDTA, pH 5.0) on a nanotating mixer overnight at 4 °C to a final concentration of 10 mg mL⁻¹. It was subsequently clarified by filtration through a 5 µm sterile filter followed by a 1.2 µm sterile filtration (Pall Corporation, Cornwall, United Kingdom), thus removing aggregates from the solution. Proteins present in ar-BSM were fractionated according to their charge on a high load 16/26 Q Sepharose High Performance anion exchange column (GE Healthcare Life Sciences, USA) installed on an Akta Avant chromatograph (GE Healthcare, Uppsala, Sweden) by elution with a high salt concentration Na-acetate buffer (10 mM Na-acetate, 1 mM EDTA, 1.2 M NaCl, pH 5.0). Fractions were analyzed by SDS–PAGE using coomassie blue- and periodic acid/schiff staining to identify fractions containing proteins/glycosylated proteins. Fractions containing BSM were pooled and dialyzed against milliQ-grade water (400:1 volume ratio) and ultimately freeze-dried. Purified BSM samples were stored at −20 °C and desiccated prior to use. All steps were performed at 4 °C to minimize proteolytic degradation. All the experimental results presented in this study were carried out exclusively with purified BSMs.

2.3. Buffer and BSM solutions

Two buffer solutions with different pH values, 3.8 and 7.4, were prepared and used throughout the study. Phosphate buffered saline (PBS) was used for pH 7.4 buffer solution, and pH 3.8 solution was prepared by addition of HCl into PBS. Purified BSMs according to the procedure described in Section 2.2 were dissolved in the respective buffer solution at the concentration of 2 mg mL⁻¹.

2.4. Substrates

Two types of substrate surfaces that are distinctively different in surface hydrophobicity, namely hydrophilic (“HL”) and hydrophobic (“HB”) surfaces were employed for the surface studies, including atomic force microscopy (AFM), optical waveguide lightmode spectroscopy (OWLS), and quartz crystal microbalance with dissipation monitoring (QCM-D). P-doped (boron) silicon surfaces with a resistivity of 10–20 Ω cm (Semiconductor Wafer Inc., Taiwan) were used in AFM studies. In order to obtain an oxide layer of approximately 30 nm, silicon surfaces were oxidized in an oxygen atmosphere [28]. The generated silica surfaces (Si/SiO₂) were cleaned by immersion for 5 min at 80 °C first in NH₄OH:H₂O₂:H₂O (1:1:5) (v/v/v) and then in HCl:H₂O₂:H₂O (1:1:5) (v/v/v) [28]. For OWLS and QCM-D experiments, SiO₂-coated OWLS sensor chips (Microvacuum, Hungary) and QCM-D sensors (model QX303, Q-Sense AB, Sweden) were used, respectively. These surfaces were cleaned in the following steps: (1) 10 min plasma treatment (in a low pressure residual air glow discharge unit, PDC–32 G, Harrick Scientific Corp., USA) (2) immersion into a Hellmanex II solution (1% volume) for 20 min (3) extensive rinsing with water (4) a second 10 min plasma treatment. After the cleaning process, silica surfaces were thoroughly rinsed with water, then with ethanol, and finally stored in ethanol until further use. All cleaned silica surfaces were rendered hydrophilic (HL) with <5° water contact angles. Hydrophobic surfaces (HB) were obtained by liquid-phase silanization of the different types of cleaned silica surfaces. Specifically, the surfaces were blown-dried with nitrogen gas and then immersed in a 0.5 mg mL⁻¹ dichlorodimethylsilane (Sigma–Aldrich, Stockholm, Sweden) in trichloroethylene solution. After silanization, the surfaces were thoroughly rinsed with trichloroethylene and then with ethanol. The water contact angle on hydrophobized silica surfaces was 95 ± 5°. HB surfaces were then stored in ethanol until use.

2.5. Dynamic light scattering (DLS)

The hydrodynamic size of BSM or its aggregates was estimated by DLS using a Zetasizer NanoZS (model ZEN3600, Malvern Instruments, UK). In this technique, the diffusion of particles moving under Brownian motion is measured, and it is converted to size and size distribution using the Stokes–Einstein relationship. Non-invasive back scatter technology (NIBS, measurement angle 173°).
is incorporated to give the highest sensitivity simultaneously with the highest size and concentration range. The light source is a He-Ne laser at 633 nm and temperature was set at 25 °C. Disposable cuvettes (PMMA, Plastibrand®) were used.

2.6. Optical waveguide lighmode spectroscopy (OWLS)

OWLS is an optical technique that detects the adsorption properties of macromolecules from solution onto the solid/liquid interface based on grating-assisted in-coupling of a plane-polarized He-Ne laser light (633 nm) into a planar waveguide (Microvacuum OWLS 120 running BioSense software version 2.6.10, Hungary). The adsorbed mass is deduced from the change of refractive index using the de Feijter formula [29]. The refractive index increment, dn/dc, of BSM solution, was measured using a refractometer (Digital Refractometer J157, Rudolph Research Analytical, Hacketts-town, NJ), and the value of 0.150 cm/g was obtained. This value is very close to that available in the literature, 0.151 cm/g [30]. Further details on the operational principles of OWLS are available in previous publications [31].

For mass uptake experiments, OWLS sensor chips (SiO2 or silanized SiO2, see Section 2.4.) were firstly exposed to buffer solutions in a flow cell until a stable baseline was obtained. The buffer solutions were supplied into the flow cell using a programmable syringe pump (Model AL1000-220, World Precision Instruments, Hertfordshire, UK) at the flow rate was 0.1 mL min⁻¹. Then, BSM solution (100 µL) was pumped into the flow cell. When BSM solution filled the flow cell, the pump was stopped, and the mass uptake was allowed for 1 h. Since the signal at this stage includes the contribution from the refractive index changes by molecules both on the surface and bulk solution, the adsorbed mass was assessed after rinsing the flow cell with BSM-free buffer solution.

2.7. Quartz crystal microbalance with dissipation (QCM-D)

QCM-D measurements were performed by using an E4 system (Q-Sense AB, Sweden). A detailed description of the technique and its basic principles can be found elsewhere [32]. Silica-coated sensors (Q-sense AB, Sweden), cleaned (hydrophilic) or hydrophobized as described above (Section 2.4.), were used. BSM solutions were supplied into the QCM-D chamber using an Ismatec peristaltic pump IPC-N 4 at a flow rate of 0.2 mL min⁻¹. When the chamber was filled with the BSM solution, the pump was stopped and the BSM was left to adsorb for 1 h under non-flow conditions. Finally, the cell was rinsed for 5 min with the corresponding BSM-free buffer solution (flow rate of 0.2 mL min⁻¹) and then stabilized for 20 min under non-flow conditions. Each experiment was performed twice, and the results are expressed as a mean and a deviation from the mean values. During the experiments, shifts in frequency, Δf, and dissipation factor, Δd, of overtones n = 3, 5, and 7 were monitored. The adsorbed mass per unit area, ΓQCM-D, as well as the shear elastic modulus, µ, and the shear viscosity η, of the films were obtained by fitting the experimental Δf and Δd with the Voigt model [33–35]. The Q-Tools software (Q-Sense AB, Sweden) was employed for fitting data to the Voigt model (initial input values for fluid density and viscosity were determined in the Voigt model [33–35]). The normal sensitivity, dΓn/dC210, of the films [36]. The lateral sensitivity of the setup, dΓn/lC0, was determined in water by the pivot method and reversed calculation [39]. The normal sensitivity, dΓn/lC0, was calculated from the slope of the deflection of the lever while pressing against a cleaved mica surface immersed in water. AFM experiments were always performed at room temperature (ca. 22 °C).

2.8. Atomic force microscopy (AFM)

A commercial AFM equipped with a liquid cell was employed (MultiMode 8 SPM with a NanoScope V control unit, Bruker AXS, Santa Barbara, CA). Rectangular silicon nitride levers with a nominal normal spring constant of 0.05 N m⁻¹ were employed (OMLCRYO800PSA, Olympus, Japan). The normal, kN, and torsional, kT, spring constants of the levers were determined with the AFM Tune IT software (ForceIT, Sweden) which uses the method developed by Sader and coworkers [37,38]. kN values were determined in the 0.059–0.061 N m⁻¹ range and kT values were in the 1.22–1.38 × 10⁻⁴ N m rad⁻¹ range. The lateral sensitivity of the setup, δf, was determined in water by the pivot method and reversed calculation [39]. The normal sensitivity, δf, was calculated from the slope of the deflection of the lever while pressing against a cleaved mica surface immersed in water. AFM experiments were always performed at room temperature (ca. 22 °C).

2.9. Nanowear measurements

The mechanical stability of BSM films was characterized at the nanoscale by means of an AFM operated in the friction force spectroscopy (FFS) mode. Briefly, in this approach, the tip of the AFM is employed to scratch, i.e. to wear, the sample surface. This is done by acquiring sets of two-dimensional scans/images on the same area of the sample. The load force applied by the AFM tip, F, is kept constant during the acquisition of each of the scans, but then varied between them. For each scan, the sample surface is scanned both in the trace and retrace directions in the contact operation mode, and the resulting topography and lateral force images for both scanning directions are recorded. The average roughness (height standard deviation) of each pair of constant-load topography images is then calculated. The average friction force, Ff, from each pair of constant-load lateral force images is also calculated as detailed in Ref. [24]. The results from AFM-based FFS experiments are represented by plotting the average sample roughness (roughness plot) and tip-sample friction force (friction plot) as a function of applied load. Specifically, the scans performed in this work consisted of 128 lines × 128 columns, and they were performed on 2 µm × 2 µm areas, while the tip velocity was set at 40 µm s⁻¹.

In AFM-based FFS (or nanowear) experiments of adsorbed protein films, the load is gradually increased from zero applied load, and depending on the nature of the sample a scan/image may be acquired where average roughness is significantly higher than that of the previous scans. This sudden increase in roughness is associated with the rupture of the film and the scan for which this occurs is termed as Rupture Scan. Further increase of the load leads, eventually, to a decrease of the roughness until a minimum value corresponding to the one obtained for the clean substrate is achieved and no further change is observed. This is associated with the complete removal of the film and the first scan for which this minimum roughness value is achieved is termed as Removal Scan. The average load and friction forces exerted during the acquisition of the Rupture and Removal Scans are associated with those needed to break and to completely remove the films, respectively. These forces were the parameters used to quantify the mechanical stability of the adsorbed BSM films in this study.

The topography of the scratched areas was visualized after nanowear experiments. For this, images of a wider area including the scratched one were obtained by operating the AFM in the contact operation mode at a low applied force (<0.5 nN) so that wear could be neglected. In this work, topography images were
represented with the WSxM software [40]. A more detailed explanation of the AFM-based FFS methodology can be found in previous publications [23,24,27].

For nanowear experiments, BSM films were prepared by pipetting 100 μL of BSM solution (2 mg mL⁻¹) onto the substrates. After 1 h, samples were gently rinsed with the corresponding BSM-free buffer solution and immediately placed on the microscope, not allowing them to dry at any moment. Nanowear experiments, including post-wear imaging, were conducted in respective buffer solution. Results are expressed as a mean and a deviation from the mean values calculated from a set of two experiments performed with different tips.

Before each experiment, tips were rubbed against a clean mica surface in order to clean and to remove asperities from their apex, a process proved crucial for obtaining reproducible results [41]. Adhesion between the tip and the BSM films was also monitored at the beginning and at the end of the experiments by means of normal force curves. In all cases, adhesion forces proved lower than those applied during nanowear experiments. Therefore, the load forces reported within this work can be considered equal to the total applied normal forces.

3. Results and discussion

3.1. Size distribution of BSM in bulk solutions

Size distribution of BSMs in pH 7.4 and 3.8 buffer solutions as determined by DLS is plotted according to the intensity in Fig. 1. These plots are obtained by averaging three measurements from each sample. In both pH solutions, the peaks corresponding to a diameter range of ca. 10–150 nm, denoted as “A” (pH 7.4) and “B” (pH 3.8), were clearly dominant. Z-average for BSM at pH 7.4 was 34.7 ± 2.1 nm. In pH 7.4 solution, peak A was nearly the single one, occupying over 99.1% of the entire area. In pH 3.8 solution, however, a second peak, denoted as “C”, corresponding to a diameter range of ca. 150–2000 nm appeared, and the areas of the main peak (“B”) and minor peak (“C”) were 87.6% and 8.5%, respectively. Z-average for BSM at pH 3.8 was 48.4 ± 10.1 nm. The maximum peak position for the main peak (A & B) was 37.8 nm for both pH solutions, while that for the second peak (C) at pH 3.8 was 531 nm. Acid-induced aggregation has been reported from porcine gastric mucins (PGM) [42,43], and it is ascribed to unfolding of hydrophobic moieties in unglycosylated N- or C-termini domains and subsequent interaction (aggregation) between them. A similar aggregation mechanism may be active for BSM too. However, as larger species tend to scatter light with much higher intensity in this technique, the number distribution of the larger species in bulk solution should be very small. Thus, despite the occurrence of larger species of BSM in acidic solution (“C” in Fig. 1), they cannot represent general structural features of BSM in this condition.

3.2. Adsorption and conformation of BSM at solid–liquid interface

3.2.1. “Dry mass” by OWLS

Representative surface adsorption profiles of BSM onto the surfaces measured by OWLS are presented in Fig. 2. In Fig. 2(a), the x-axis represents the time and y-axis the adsorbed mass. The time “0” refers to the point of injecting BSM solution into the flow cell. The adsorption experiment was duplicated for both solutions (pH 7.4 vs 3.8) and substrates (HL vs HB). The mean values of the results and error bars are presented in Fig. 2 and Table 1. In all cases, BSM adsorbed fairly fast. Upon injection of the BSM solutions, a rapid increase of the mass uptake was followed by a plateau within ca. 20 min. BSM adsorbed more onto hydrophobic than hydrophilic substrates. Additionally, BSM adsorbed more from acidic pH (3.8) than neutral pH (7.4), and this pH-dependence was stronger for hydrophilic than for hydrophobic substrates. Overall, the adsorbed mass was ranked as HB-pH 3.8 > HL-pH 3.8 ≈ HB-pH 7.4 > HL-pH 7.4. It is worth to mention that the validity of the substrates chosen in this study, i.e. clean and methylated silica, for studying the effect of substrate hydrophobicity on the interfacial behavior of biological molecules is well-established from many previous studies [44–46].

Variation in the adsorbed mass of BSM as a function of pH and substrate hydrophobicity can be understood in terms of the interaction between BSM molecules (pH effect) as well as the

![Fig. 1. Intensity vs hydrated diameter plots of BSM in pH 7.4 and 3.8 solution. “A” represents the major peak for BSM at pH 7.4, and “B” and “C” represent the major and minor peaks for BSM at pH 3.8.](image1)

![Fig. 2. (a) Representative surface adsorption profiles of BSM, and (b) Average adsorbed mass, for different surface hydrophobicity and solution pH as characterized by OWLS. The concentration of BSM solution was 2 mg mL⁻¹. The surface adsorption was allowed for 60 min.](image2)
interaction between BSM and the substrate (pH effect and substrate hydrophobicity effect). Silica substrates (isoelectric point of ca. 2) are negatively charged in neutral aqueous solution, and thus electrostatic repulsion between the substrate and BSM molecules is mainly responsible for the retarded adsorption onto the surface [47–49]. As the pH is lowered, their charge is reduced because of partial protonation. In contrast, the nonpolar characteristic of the hydrophobized substrates would not be influenced by pH [44]. BSM is expected to be negatively charged at both pHs, but the net charge should be much higher at neutral pH as its isoelectric point is ca. 2–3 [48]. Moreover, BSM contains hydrophobic patches on both the C- and N-termini which could be involved in attractive interactions with hydrophobic surfaces [50]. Generally, adsorption of amphiphilic polymers onto a nonpolar surface is limited by steric repulsion, and for charged amphiphilic polymers [51], this limitation is exacerbated by the electrostatic repulsion in order to minimize the accumulation of charges on nonpolar surface. This explains the more favorable adsorption of BSM for the case of HB-pH 3.8 compared to HB-pH 7.4. The lowest adsorbed mass observed on hydrophilic substrates at pH 7.4 can be explained by taking into account that both substrates and BSMs bear a high net negative charge. In this case, the interactions responsible for the formation of the film should be either hydrogen bonding or electrostatic attractions involving the positively charged BSM residues. The higher mass observed on hydrophilic substrates at low pHs. HL-pH 3.8, can easily be explained by a reduction of the repulsive electrostatic interactions.

### 3.2.2. “Wet” mass by QCM-D

The time evolution of the adsorbed mass per unit area, \( \Gamma_{\text{QCM,D}} \), and \( G'/G' \) ratios for representative QCM-D experiments consisting of BSM adsorption and subsequent rinsing on both substrates, i.e. HL and HB, and pH conditions, i.e. pH 7.4 and pH 3.8, studied in this work are shown in Fig. 3 (the corresponding plots for shear elastic modulus, \( \mu \), and shear viscosity modulus, \( \eta \), are shown in Fig. S2 in the Supplementary Data). When analyzing these data, it is important to note that in contrast to OWLS which probes the dry mass of the adsorbed films, QCM-D probes the mass of the solvent molecules that are strongly associated with the adsorbed film as well [52]. Therefore, we refer to the mass measured by means of QCM-D as “wet” mass in contraposition to the “dry” mass measured by means of OWLS.

Similarly with OWLS, QCM-D also showed a fast initial adsorption of BSM onto the different substrates. In the HB-pH 7.4 and HB-pH 3.8 cases, a large initial overshoot was initially observed, although the disappearance of the overshoot was much faster in the case of HB-pH 3.8. Similar overshoots have been previously reported [33]. They may indicate some desorption or conformation changes of the molecules within the films. Since there is no desorption of adsorbed BSM molecules within this period, i.e. prior to rinsing with buffer, as observed by OWLS (Fig. 2), “self-desorbing” wet-mass can be entirely attributed to solvent, i.e. water. Much slower desorption kinetics of solvent from BSM layers from HB-pH 7.4 reflects their superior capability to interact with water due to higher charge and more extended structure. The wet mass, \( \Gamma_{\text{QCM,D}} \), was observed to be in the order of HB-pH 7.4 > HB-pH 3.8 > HL-pH 3.8 > HL-pH 7.4. In all the cases, the water content of the films is substantial and given as weight fraction in Table 1.

It is noted that the highest wet mass was observed from HB-pH 7.4 even though the highest dry mass was obtained from HB-pH 3.8. This means that, although less BSM adsorbs onto HB substrates at pH 7.4 than at pH 3.8, it adopts a conformation that attracts more solvent at pH 7.4 than at pH 3.8. One possible explanation is that, on HB substrates, BSM film adopts a more compact conformation at pH 3.8, incorporating relatively less amount of solvent. This would be firstly due to the reduced intra- and intermolecular repulsion, following the neutralization of charged moieties. Further, the affinity of water to BSM is also expected to be lower when

<table>
<thead>
<tr>
<th>Liquid</th>
<th>Substrate</th>
<th>( \Gamma_{\text{QCM,D}} ) (mg m(^{-2}))</th>
<th>Water content (mg m(^{-2}))</th>
<th>Water content (w/w)</th>
<th>( G'/G' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>HL SiO(_2)</td>
<td>0.07 ± 0.1</td>
<td>3.43</td>
<td>0.98</td>
<td>0.59 ± 0.04</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>HB SiO(_2)</td>
<td>1.14 ± 0.24</td>
<td>11.06</td>
<td>0.91</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td>PBS</td>
<td>HL SiO(_2)</td>
<td>1.20 ± 0.09</td>
<td>6.30</td>
<td>0.84</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>pH 3.8</td>
<td>HB SiO(_2)</td>
<td>1.66 ± 0.43</td>
<td>9.48</td>
<td>0.83</td>
<td>0.66 ± 0.04</td>
</tr>
</tbody>
</table>

![Fig. 3. Time evolution of (a) Voigt mass and (b) \( G'/G' \) ratio for the adsorption of BSM (the concentration = 2 mg mL\(^{-1}\)) on hydrophobized silica in PBS pH 3.8 (black/triangles), on hydrophobized silica in PBS pH 7.4 (red/squares), on hydrophilic silica in PBS pH 3.8 (green/diamonds) and on hydrophilic silica in PBS pH 7.4 (blue/circles). \( t = 0 \) correspond to the time point when proteins were injected. Rinsing with respective protein-free buffer was performed 60 min after adsorption onset. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](#)
BSM is neutralized (acidic pH) than charged (neutral pH), and thus BSM is less hydrated at acidic pH.

The wet mass of BSM on HL substrates is significantly lower than that found on HB substrates, following the same trend observed for the dry mass. Moreover, it is notable that at pH 7.4, the relative amount of solvent coupled to the films is exceedingly higher than that found for any other conditions investigated. Considering the substantially smaller amount of adsorbed dry mass of BSM under HL-pH 7.4 condition, this implies that BSM molecules are highly stretched due to extremely high hydration.

Interestingly, the differences observed in the relative amount of coupled water between the different conditions investigated were not accompanied by any significant difference in the viscoelasticity of the systems. As shown in Fig. 3b, the $G'/G$ ratios for all the investigated cases were within a similar range, viz. 0.59–0.66, indicating that the elastic component of the films overpassed their viscous one.

3.3. Nanowear properties of BSM films

Results from representative nanowear experiments on BSM films are shown in Fig. 4. In these experiments, the interval of load change, both in increase and decrease, was ca. 0.2 nN between scans/images. For comparison, the roughness and friction forces measured from a clean substrate (no BSM adsorbed) with the same AFM tip are also presented. From the measured roughness and friction plots, we have determined the load and friction forces needed both to break and remove the BSM films, and used these quantities to characterize their mechanical stability. It is important to bear in mind that these quantities are dependent on factors such as the size and shape of the tip. Nevertheless, the ratio between these forces measured on different systems can be considered as an indicator of the differences between their mechanical stability. We have thus plotted (Fig. 5) the load and friction forces needed both to break and to remove BSM films in each condition as normalized by those of the BSM films of HB-pH 3.8 as reference.

3.3.1. HB substrates

BSM films formed on HB substrates exhibited a similar qualitative response to the scratch both at pH 7.4 and at pH 3.8. At the beginning of the experiments, i.e. under the lowest applied loads (<2 nN for the case of pH 3.8 and <0.5 nN for the case of pH 7.4), a low and fairly constant roughness, i.e. characteristic of a planar topography, was observed. This is indicative of a non-destructive sliding of the tip along the intact BSM films. In this regime, the friction forces on BSM films were lower than those on bare substrates. The load and friction forces for the Rupture Scan at pH 7.4 ($F_L = 0.69 \pm 0.09$ nN and $F_F = 0.79 \pm 0.18$ nN) were determined to be lower than those at pH 3.8 ($F_L = 2.53 \pm 0.17$ nN and $F_F = 3.10 \pm 0.21$ nN). Further increase of the load identified that...
the loads and friction forces for the Removal Scan at pH 7.4 ($F_L = 1.85 \pm 0.22$ nN and $F_F = 1.79 \pm 0.47$ nN) were substantially lower than those at pH 3.8 ($F_L = 5.72 \pm 0.41$ nN and $F_F = 6.50 \pm 0.23$ nN) as well. Therefore, BSM films formed on HB substrates exhibited a higher mechanical stability (wear resistance) at acidic (pH 3.8) than at neutral (pH 7.4) pH conditions. This is associated with, and most probably originating from, the more compact conformation revealed for BSM films at low pHs by QCM-D experiments (vide supra).

It is also noted that, except for the initially low applied load regime (<2 nN), the scans/images on the BSM films exhibited higher average friction forces than those on bare HB substrates at both pH conditions. This difference in friction persisted even when the load started to be lowered after the complete removal of the BSM films. However, friction increased with load at a similar rate on both surfaces (substrates with or without BSM films), suggesting that in both cases the tip is interacting with the underlying HB substrate. This supports that, after the Removal Scan, the BSM films were effectively removed and not, for instance, pressed down. The difference in friction force measured between BSM-coated and bare HB substrates is similar to that observed in previous nanowear studies of different proteinaceous layers [23,27] where it was attributed to the ability of the proteins to laterally diffuse on the underlying substrates; in this high-load regime, the tip is able to sweep away the diffusing molecules, but at the cost of exerting a higher friction force compared to the case with no diffusing molecules on the surface. We believe that this mechanism applies in the case of BSM molecules on HB substrates as well. Lack of hysteresis between the friction forces and roughness data collected during increasing and decreasing the load between scans further supports this scenario. Eventually, lowering the load led to a reestablishment of the sample roughness. This was accompanied by a gradual decrease in friction forces down to values similar to those measured in the beginning of the experiment when the films were still not broken. In other words, friction vs load plots were reproduced almost in a reverse way. The recovery of the BSM films formed on HB substrates is also confirmed by the topography images obtained after the experiments (Fig. 4c and f), which show no significant difference in height between the scratched and the non-scratched areas of the BSM films on surfaces. It cannot be ruled out that the lateral diffusion may be facilitated by either the scanning itself, which could provide energy to the molecules scratched toward the edges of the scanned areas, or by the accumulation of these molecules at these edges being energetically unfavorable. Nevertheless, the experimental data unequivocally proves the ability of BSM molecules to laterally diffuse on HB substrates. This ability can be reasonably attributed to the range of the hydrophobic forces (which mediate molecule–substrate interactions) being larger than the separation between anchoring sites [53,54]. It is fair to note that, whereas lateral diffusion of mucins within gel-like networks has been previously reported [55], we present for the first time experimental evidences showing the lateral diffusion of mucins at solid–liquid interfaces.

3.3.2. HL substrates

The results obtained for the BSM films formed on HL substrates differ substantially from those obtained for films formed on HB substrates. Removal Scans are clearly identified for the BSM films formed on HL substrates in pH 3.8.
formed on **HL** substrates whereas **Rupture Scans** are not. The load and friction forces needed to remove the films at the two investigated pH conditions were fairly similar; \( F_1 = 0.88 \pm 0.07 \text{nN} \) and \( F_2 = 0.47 \pm 0.12 \text{nN} \) at pH 7.4 and \( F_1 = 1.04 \pm 0.28 \text{nN} \) and \( F_2 = 0.83 \pm 0.13 \text{nN} \) at pH 3.8. From these results, it is inferred that BSM films are significantly more stable when formed on **HB** substrates. As commented, the interaction between BSM molecules and hydrophilic substrates is probably mediated by hydrogen bonding or electrostatics involving the positively charged BSM residues. Both of these interactions are presumably lower than the hydrophobic interactions between BSM molecules and **HB** substrates, this being the origin of the difference in mechanical stability.

In contrast to what was observed from the films on **HB** substrates, friction plots registered for the films on **HL** substrates overlapped with those measured for clean bare **HL** substrates. This suggests that BSM molecules do not diffuse on the **HL** substrates on the experimental time scale. This is supported by the soft AFM imaging of the scratched areas. These images (Fig. 4i and l) show clear differences between the inner scratched area and the outer non-scratched area indicating that the film coverage is not recovered after the experiments [56]. The absence of lateral diffusion is not surprising since, as previously commented, the molecule–substrate interaction is mediated by hydrogen bonding or electrostatic forces in this case. Screening effects by the relatively high ionic strength used in our experiments result most probably in the range these interactions being shorter than the distance between anchoring points [57–59].

The clear identification of film removal by topographic images implies that the rupture-like event does occur, despite the lack of a **Rupture Scan** for BSM films on **HL** substrates. As the topography images showed, the intact BSM films formed on **HL** substrates are significantly rougher than those formed on **HB** substrates, which is presumably related to relatively lower adsorbed amount of BSM and the formation of island-like features on the surface. Thus, identification of the rupture of the films by probing an increase in their roughness is not feasible. It could be also that films formed on **HL** substrates are so weak that they break at forces lower than those applied in our experiments.

### 3.3.3. Human saliva vs BSM

The role of mucins, one of the main components of salivary films, in the lubrication between saliva-coated surfaces is well-known. However, their role in the protective function offered by salivary films has still not been determined. The results from the present work shed light on this aspect. In recent studies [23,24], the mechanical stability of salivary films formed both on **HL** and **HB** substrates has been characterized, also at different pH conditions. While those studies were performed at lower ionic strengths compared to the present study, it may be still useful to compare the data of their results with those presented in this work. The mechanical stability measured for BSM films on **HB** substrates in PBS pH 7.4 is slightly lower than that measured for salivary films adsorbed on the same type of substrate in water with a pH in the range of 5–7, but is still within the same order of magnitude. By lowering pH, on the other hand, the mechanical stability of salivary films formed on **HB** substrates decreases whereas that of BSM films on similarly hydrophobic substrates increases. Larger differences were observed from the behavior on **HL** substrates; at both pH conditions, the forces needed to remove salivary films were at least one order of magnitude higher than those needed to remove BSM films. Thus, our results suggest that mucins cannot fully represent the protective function of salivary films under high pressure. This role should then be attributed to other proteins, or to the synergetic action of mucins with other proteins.

### 4. Conclusions

In this study, we have generated BSM films under variation of solution pH and substrate hydrophobicity, and characterized adsorption, conformation, and nanowear properties at the solid–liquid interface. DLS studies revealed the occurrence of larger species (ca. 150–2000 nm in hydrodynamic size) upon lowering pH from 7.4 to 3.8, but only at a very small ratio. BSM adsorption at the solid–liquid interface was characterized by means of OWLS for “dry” adsorbed mass and of QCM-D for “wet” adsorbed mass. While the dry mass was higher on hydrophobic substrates at acidic pH (**HB**–pH 3.8), the wet mass was higher on hydrophobic substrates at neutral pH (**HB**–pH 7.4). The lowest amount of adsorbed molecules was observed for hydrophilic substrates at neutral pH (**HL**–pH 7.4). Generally, hydrophobic substrates tend to attract more BSM molecules onto the surfaces and neutral pH tends to facilitate more hydration of the adsorbed BSM molecules. AFM operated in the FFS mode proved to be suitable for studying the mechanical stability, in particular the anti-wear properties, of BSM films on a nanoscale level. Nanowear studies showed that BSM films formed on **HB** substrates are significantly stronger than those formed on **HL** substrates. This correlates with the higher amount of BSM adsorbed on **HB** substrate and is presumably attributed to the occurrence of attractive molecule–substrate hydrophobic interactions. BSMs form not only stronger films, but also show the ability to laterally diffuse on **HB** substrates. This enhances the protective role of the films as damage is almost immediately healed after pressure reduction. Moreover, BSM films on **HB** substrates become stronger at acidic conditions. Based on the structural properties of BSM films obtained from OWLS and QCM-D studies, it is rationalized that the enhanced mechanical stability of BSM films at pH 3.8 compared to pH 7.4 on **HB** surfaces originates from stronger inter- and intra-molecular packing (interaction) due to reduced electrostatic repulsion. Finally, when comparing the mechanical stability data of BSM films presented here with that previously reported for human salivary films, it is inferred that mucins alone do not fully account for the mechanical strength of salivary films.

### Acknowledgments

This study was supported by Malmö University and European Research Council (ERC). Thomas Arnebrant acknowledges the Gustaf Th. Ohlsson foundation. Seunghwan Lee and Jan Busk Madsen acknowledge European Research Council (ERC) for the financial support (Funding scheme, ERC Starting Grant 2010, and the project number 261152). Professor Dr. Birte Svensson and Professor Dr. Maher Abou Hachem, Department of Systems Biology, Technical University of Denmark, are appreciated for their support in purification of BSM. Professor Anders Baun and Ms. Denisa Cupi (Department of Environmental Engineering, DTU) are appreciated for their assistance with DLS measurements.

### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.jcis.2014.04.058](http://dx.doi.org/10.1016/j.jcis.2014.04.058).

### References

The anchoring points on HL (silica) surface would be the exposed silanol groups. Given that not all silanol groups will be ionized, the separation between the length will be below 1nm, and thus similar to (or most probably shorter than, 0.1 nm⁻² range [58, 59]. At the experimental conditions (PBS) the Debye length will be below 1nm or less. This length is much smaller than those characterizing hydrophobic interactions, which can be in the order of tens of nanometers.

It has been shown that the methylation procedure used for the hydrophobization of silica surfaces does not significantly change the zeta potential of the surfaces [45]. Moreover, it has been shown that such a modification does not alter surface roughness at least in the nm order [46]. Thus, the only relevant difference between clean and methylated silica surfaces is that the methylated ones will interact with the adsorbed molecules via hydrophobic interactions as well, which validates the employment of these surfaces to study the effect of the substrate hydrophobicity on the interfacial behavior of BSM molecules.

Retarded adsorption of BSM onto SiO₂ surface at neutral pH due to electrostatic repulsion should not be generalized to all hydrophobic surface though. For example, mica is a representative exception, where the surface is extremely hydrophilic and overall surface charge is negative, yet the adsorption of BSM [48,49] or other mucin [21] is feasible. Thus, the notation of “hydrophobic (HL)” surface in this study should be strictly speaking, limited to SiO₂ surface.

The anchoring sites on HB (methylated silica) surfaces will be the exposed methyl groups. It is difficult to speculate on the density of exposed methyl groups on the HB surfaces. It is nevertheless reasonable to expect that they would saturate the surface as higher contact angles could not be experimentally achieved (even though the silanes initially bind the exposed silanol groups on the HB surfaces. It is nevertheless reasonable to expect that they would saturate the surface). In this scenario, the separation between exposed methyl groups will not be much longer than the size of a silane molecule, i.e. 1nm or less. This length is much smaller than those characterizing hydrophobic interactions, which can be in the order of tens of nanometers.

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The anchoring points on HL (silica) surface would be the exposed silanol groups. Reported surface density values for these groups usually lie in the 1-10 nm⁻² range [58, 59]. At the experimental conditions (PBS) the Debye length will be below 1nm and thus similar to (or most probably shorter than, given that not all silanol groups will be ionized) the separation between the anchoring silanol groups.

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Appendix 2: Conformation of Bovine Submaxillary Mucin (BSM) Layers on Hydrophobic Surface As Studied by Biomolecular Probes

In this study we were interested in determining whether antibodies specific for either the terminal domains or the central glycosylated domains could be employed to monitor differences in the conformational anchoring onto the surface as a function of concentration. The manuscript has been submitted to International Journal of Biological Macromolecules.

My contribution to this study was supplying purified BSM and writing the experimental part for the technique.
Abstract: In the present study, the conformational changes of bovine submaxillary mucin (BSM) adsorbed on a hydrophobic surface (polystyrene (PS)) as a function of concentration in bulk solution (up to 2 mg/mL) have been investigated with biomolecular probe-based approaches, including bicinchoninic acid (BCA), enzyme-linked immunosorbent assay (EIA), and enzyme-linked lectin assay (ELLA). The conformation and hydrodynamic diameter of highly purified BSM molecules, as characterized by circular dichroism (CD) spectroscopy and dynamic light scattering (DLS), respectively, showed a slight, yet gradual coiling and compaction in response to the increase in BSM concentration in bulk solution. Adsorbed masses of BSM onto hydrophobic surface, as probe by BCA, showed a continuously increasing trend up to 2 mg/mL. But, the signals from EIA and ELLA, which probe the concentration of available unglycosylated C-terminals and the central glycosylated regions, respectively, showed complicated non-linear responses with increasing surface concentration. The results from this study support the conventional amphiphilic, triblock model of BSM in the adsorption onto hydrophobic surface from aqueous solution. The biomolecular probe-based approaches employed in this study, however, provided further details on the conformational changes of BSM on surface, in particular the accessibility of glycosylated and unglycosylated domains with increasing surface concentration.
Abstract

In the present study, the conformational changes of bovine submaxillary mucin (BSM) adsorbed on a hydrophobic surface (polystyrene (PS)) as a function of concentration in bulk solution (up to 2 mg/mL) have been investigated with biomolecular probe-based approaches, including bicinchoninic acid (BCA), enzyme-linked immunosorbent assay (EIA), and enzyme-linked lectin assay (ELLA). The conformation and hydrodynamic diameter of highly purified BSM molecules, as characterized by circular dichroism (CD) spectroscopy and dynamic light scattering (DLS), respectively, showed a slight, yet gradual coiling and compaction in response to the increase in BSM concentration in bulk solution. Adsorbed masses of BSM onto hydrophobic surface, as probe by BCA, showed a continuously increasing trend up to 2 mg/mL. But, the signals from EIA and ELLA, which probe the concentration of available unglycosylated C-terminals and the central glycosylated regions, respectively, showed complicated non-linear responses with increasing surface concentration. The results from this study support the conventional amphiphilic, triblock model of BSM in the adsorption onto hydrophobic surface from aqueous solution. The biomolecular probe-based approaches employed in this study, however, provided further details on the conformational changes of BSM on surface, in particular the accessibility of glycosylated and unglycosylated domains with increasing surface concentration.
Marjam Ott  
marjam.ott@angstrom.uu.se

Head of the Cell Laboratory  
Applied Materials Science,  
Uppsala University  
Box 534, S-751 21 Uppsala, Sweden

Bruno Zappone  
bruno.zappone@fis.unical.it

Liquid Crystal Laboratory (LICRYL)  
Istituto per i processi Chimico-Fisici (IPCF)  
Consiglio Nazionale delle Ricerche (CNR)  
c/o Dipartimento di Fisica  
Università della Calabria  
Ponte P. Bucci, cubo 33B  
87036 Arcavacata di Rende (CS), Italy

Andrew Round  
a.round@uea.ac.uk

Telephone: +44 (0) 1603-593392  
School Position: Lecturer in Pharmaceutical Nanosciences  
University of East Anglia

Thomas Arnebrant  
thomas.arnebrant@mah.se

Malmö University  
Faculty of Health and Society,  
205 06 Malmö, Sweden  
Telephone: 040-66 57927
Gleb Yakubov
g.yakubov@uq.edu.au

School of Chemical Engineering
Faculty of Engineering, Architecture and Information Technology
The University of Queensland

Ibraheem A. Bushnak
bushnak@ualberta.ca

ECERF 7-037-05E
Department of Chemistry
University of Alberta
Phone: 780-710-4757
Conformation of Bovine Submaxillary Mucin Layers on Hydrophobic Surface
As Studied by Biomolecular Probes

Kirsi I. Pakkanen, Jan B. Madsen, and Seunghwan Lee*

Department of Mechanical Engineering, Technical University of Denmark, DK-2800, Kgs. Lyngby, Denmark

Key words: BSM; EIA; ELLA

*Corresponding author: Seunghwan Lee, seele@mek.dtu.dk, Tel) +45 4525 2193, Fax) +45 4593 6213

Abstract

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adsorption onto hydrophobic surface from aqueous solution. The biomolecular probe-based approaches employed in this study, however, provided further details on the conformational changes of BSM on surface, in particular the accessibility of glycosylated and unglycosylated domains with increasing surface concentration.

1. Introduction

Mucins are large, high-molecular weight glycoproteins that constitute the main macromolecular component of mucus, a slimy layer that hydrates and protects the interface between the body and the exterior environment [1-3]. Mucins comprise a large glycoprotein family that includes a number of mucin subtypes which share considerable degree of sequence homology, yet display differences too in their glycosylation, physical properties, and functions [1,4]. While mucins are extremely complex, heterogeneous, and hierarchical in their composition and structure, they are often simplified to an amphiphilic triblock copolymer model, such as “Pluronic®” [5,6]; a long linear polypeptide backbone with central, heavily O-glycosylated domain (thus hydrophilic) and “naked”, unglycosylated C- and N-termini (possibly hydrophobic) domains.

As biological functions of mucins take place predominantly at interfaces, mucins are very interesting molecules for surface science and engineering. For instance, mucins are an indispensable component of saliva replacements [7,8] due to their slippery nature [6,9]. In a related, yet broader context, spontaneous adsorption and assembly of mucins onto surfaces of synthetic materials have become an interesting topic. In the adsorption of mucins onto hydrophobic surfaces from aqueous solution, it is often proposed that unglycosylated C- and N-terminal domains act as anchor blocks due to the hydrophobicity, whereas the glycosylated central domain stretches out to interact with bulk water as buoyant blocks [10-12]. This view is similar to that on the interactions of another glycoprotein from synovial joints, lubricin, with surfaces [13,14]. This model of mucin interactions
with surfaces provides a very useful picture on the conformation of mucins at the interface, and can be used to explain various experimental observations, for example, conversion of hydrophobic surfaces to hydrophilic one [5,11], an effective lubrication at hydrophobic interface in aqueous environment [6,9].

However, amphiphilic triblock copolymer model is based on the structural and compositional features of mucins, not direct probe of the actual conformation of mucins on the surface. Due to its long and flexible structural features, mucins are readily capable of adjusting their packing density and conformation on surface in response to the changes in concentration, ionic strength, and pH, and thus, higher-level of complexity in conformation is expected than synthetic amphiphilic molecules. In this context, we propose that antibody- and lectin-based assays may broaden our understanding on the conformation of mucins on surface [15] based on the direct interaction of antibody or lectin with different parts of mucins on surface, namely terminal regions (“naked” peptides) and central regions (glycans), respectively. In this study, we have tested the feasibility of this approach to investigate the conformational change of highly purified bovine submaxillary mucin (BSM) layers on a hydrophobic surface via spontaneous adsorption from aqueous solution as a function of concentration of the bulk BSM solution.

2. Material and methods

2.1 Chemicals

Two biotinylated lectins (Triticum vulgaris lectin, wheat germ agglutinin (WGA) and Arachis hypogaea lectin peanut agglutinin (PNA)), streptavidin-conjugated horse radish peroxidase (Strp-HRP), and 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate for HRP were from Sigma Aldrich (St. Louis, MO). Anti-MUC19 antibody was purchased from Abcam (Cambridge, UK) and biotinylated anti-goat secondary antibody was from Sigma Aldrich (St. Louis, MO). All buffer ingredients were
from Sigma Aldrich (St. Louis, MO). Water used in the study was ultra-pure grade and purified with a Millipore Direct-Q system (Merck Millipore, Billerica, MA).

2.2 Bovine submaxillary mucin (BSM)

Bovine submaxillary mucin (BSM; type I-S, M3895) was purchased from Sigma Aldrich (St. Louis, MO). To remove impurities, anion exchange chromatography was employed to further purify the BSM. The purification procedure was modified from the method proposed by Sandberg et al. [16] and is described in detail elsewhere [17]. Briefly, BSM was dissolved in Na-acetate buffer (10 mM Na-acetate, 1 mM EDTA, pH 5.0) at 4 °C to a final concentration of 10 mg/mL, filtered through a 5 µm sterile filter (Pall Corporation, Cornwall, U.K.), and fractionated on a High Load 16/26 Q Sepharose High Performance anion exchange column (GE Healthcare Life Sciences, USA) by using Na-acetate buffer as eluent. Purity of the obtained BSM fractions was analyzed with SDS-PAGE (data not shown). The fractions containing BSM were pooled, dialyzed against water, and freeze-dried, and kept in freezer (-20 °C) until use.

2.3 Dynamic light scattering (DLS)

Dynamic light scattering was employed to characterize the hydrodynamic diameter of BSM molecules as a function of concentration in bulk solution. PBS buffer was filtered (0.22 µm) prior to preparation of BSM solution. Malvern Zetasizer Nano ZS two angle particle and molecular size analyzer (Malvern Instruments, Worcestershire, UK) were used for DLS. The measurements were performed at 25 °C and each sample was measured in triplicates. The Malvern Zetasizer software was used to analyze the obtained data.

2.4 Circular dichroism (CD) spectroscopy
Far-UV CD spectra of BSM dissolved in different concentrations in PBS buffer were measured using a rectangular quartz cuvette with either 1 mm (BSM concentrations 0.25 – 1 mg/mL) or 0.5 mm (BSM concentration 2 mg/mL) path length (Hellma GmbH & Co. KG, Müllheim, Germany) using a Chirascan spectrophotometer (Applied Photophysics Ltd, Surrey, UK). Temperature was maintained at 25 °C using a CS/PCS single cell Peltier temperature controller (Applied Photophysics Ltd, Surrey, UK). The spectra were recorded from 280 to 195 nm with step size of 1 nm and bandwidth of 1 nm. CD signals of the PBS background were subtracted from the data. The measured ellipticity (mdeg) was converted to molar ellipticity (deg cm$^2$/dmol) based on equation:

$$[\theta] = \theta/1000 \cdot c \cdot 100 \cdot l$$

where $\theta$ is the measured ellipticity in mdeg, $c$ concentration of BSM in mol/L and $l$ the path length of the cuvette in cm. Molarity of BSM in the cuvette was based on the assumption of total molecular weight of 2 MDa [16] and assumption that protein backbone constitutes 36% of the molecular weight [18,19].

2.5 BSM coatings for plate assays

BSM coatings were formed on hydrophobic 96-well “Lipo Grade” microtiter plates (Brand GMBH + CO KG, Wertheim, Germany). Static water contact angle of the well bottom surfaces was 96.5 ± 3.3 ° as measured with goniometer (Ramé-hart, Model 200 Std G, Netcon, NJ). The purified BSM was dissolved in PBS and diluted for appropriate concentrations ranging from 0 to 2 mg/mL. The samples were allowed to adsorb onto the well surface overnight at +4 °C. Next day, the BSM solutions were removed and the wells were washed with PBS.
2.6 BCA Protein quantification assay

Amount of proteins in the coated wells was estimated using a commercial bicinchoninic acid (BCA) protein quantification assay kit (Sigma Aldrich). The assay is based on the reduction of Cu$^{2+}$ ions to Cu$^{+}$ in the presence of peptide bonds (proteins) and further, complex formation between Cu$^{+}$ with BCA to form a colorful product [20]. The assay was performed according to the manufacturer’s instruction. The microtiter wells were exposed to BSM solution overnight (ca. 18 hrs) at 4 °C.

2.7 Sequence comparisons of mouse and bovine MUC19

To ensure the specificity of anti-mouse MUC19 antibody with BSM (MUC19), the sequence of antibody target was compared with that of bovine MUC19 using the BLAST network service at the Swiss Institute of Bioinformatics (SIB). The SIB BLAST network service uses a server developed at SIB and the NCBI BLAST 2 software [21].

2.8 Enzyme-linked immunosorbent assay (EIA)

Antibody to MUC19 (BSM) C-terminus (amino acids 7461-7474: ECKRSVKYNYETFQ) was used in EIA experiments to detect the unglycosylated polypeptide ends of BSM. The monoclonal antibody to MUC19 was used as primary antibody and biotinylated anti-goat as secondary antibody. Streptavidin-HRP was used to detect the biotinylated secondary antibody. All detection steps were performed in Tris-buffered saline (TBS) with 0.2% Tween 20. TMB was used as a substrate for HRP and the enzymatic reaction was stopped with 1 M hydrochloric acid. Absorbance of the yellow end-product at 450 nm was measured using a plate reader (ELx800, BioTek Instruments, Winooski, VT). The results were corrected for the background of unspecific absorbance, and the intensities
were normalized to PBS which was set to zero absorbance. The real absorbance of PBS controls was typically not higher than that of unspecific background (blank controls).

2.9 Enzyme-linked lectin assay (ELLA)

In ELLA experiments, biotinylated lectins (see details in section “2.1 Chemicals”) were used to detect the glycosylated chains of BSM. The lectins were allowed to interact with BSM bound on the wells and streptavidin-HRP was used to detect the biotinylated lectins similar to EIA assay described above. Enzymatic reaction with HRP, absorbance measurements and data analysis were done as described for EIA assay above.

3. Results & Discussion

3.1 BSM in bulk solution: hydrodynamic size and secondary structure

The distribution of hydrodynamic diameter, $D_h$, of BSM molecules as characterized by DLS is presented in Figure 1.

![Graph](Image)

**Figure 1.** Hydrodynamic diameter, $D_h$, of BSM in PBS at different concentrations by dynamic light scattering (DLS) in the bulk concentration range from 0.01 mg/mL to 2.0 mg/mL. The presented numbers are averages of 3 measurements and error bars represent standard deviation (SD). It is noted that in low concentration regime (0.01 m̄ 0.015 mg/mL, grey symbols), the validity of the values is weak due to low count rate.
The $D_h$ of BSM was in the range of ca. 61.8 – 35 nm (Z average) over the concentration range from 0.01 mg/mL to 2 mg/mL. In very low concentration regime, such as 0.01 – 0.015 mg/mL, though, the accuracy of data is insufficient due to too low count rate (on the level of 30 – 40 kcps, which is only ca. 10% of the other measurements at higher concentrations). Within the concentration range from 0.15 mg/mL to 2 mg/mL, $D_h$ showed ca. 10% decrease from 39 nm to 35 nm. With or without taking the data in the low concentration regime (0.01 – 0.015 mg/mL) into account, a slight, yet gradual decrease in the $D_h$ with increasing concentration is clear. This means that no aggregation between BSM molecules is expected with increasing concentration up to 2 mg/mL. Instead, the decrease of $D_h$ with increasing bulk concentration can be interpreted as a compaction between BSM molecules.

Far-UV CD spectra obtained from BSM at varying concentration shared an overall form indicative of random coil structure with a characteristic strong negative band at around 200 nm and a slight positive shoulder at 220 nm (Figure 2A).

![Figure 2](image)

**Figure 2.** Analysis of BSM conformation at different concentrations using circular dichroism spectroscopy. (A) Far-UV CD spectra of BSM at 0.25 mg/mL (light grey line), 0.5 mg/mL (grey), 1 mg/mL (dark grey) and 2 mg/mL (black). The spectra are averages of three measurements at each concentration. (B) Molar ellipticity at 205 nm as a function of BSM concentration. Error bars represent standard deviation (SD).
With increasing concentration, the 200 nm negative band shifted slightly towards higher wavelength and became more intense (Figure 2A) which could also be caused by the compaction as mentioned above. To have a clearer view on the changes, the molar ellipticity at 205 nm was plotted as a function of BSM concentration. Wavelength 205 nm was selected as an analysis wavelength, as the signal-to-noise ratio at 200 nm was hampered by the ions in buffer solution. The plot shows that negative band characteristic of random coil structure at around 200 nm is intensified with increasing concentration of BSM (Figure 2B). Overall, DLS and CD spectroscopy indicate that no intermolecular aggregation is occurring between BSMs in the concentration range up to 2 mg/mL.

3.2 Adsorbed amount of BSM on a hydrophobic surface: BCA

BCA protein quantification assay was employed to characterize the amount of BSM adsorbed on a hydrophobic surface. As expected, the mass of BSM adsorbed on the hydrophobic microtiter wells was proportionally, yet non-linearly, increasing with the concentration of BSM introduced to the wells (Figure 3A).

![Figure 3](image-url)

**Figure 3.** Adsorbed masses of BSM on well surfaces as characterized with BCA protein quantification method. (A) Amount of protein (BCA detection), (B) estimated amount of BSM (protein and glycan) on well surfaces as function of BSM amount introduced to the wells. The data are averages of 3 BCA measurements at each concentration. The error bars represent standard deviation (SD). The numbers are normalized to PBS control = 0 µg/well.
As mentioned above, BCA is based on the detection of peptide bonds in proteins [20], and thus the molecular weight of glycans of mucins is excluded. Taking the model proposed by Bettelheim et al. that the weight of protein part is 36% [18,19], the total masses of BSM molecules adsorbed onto the well surfaces were estimated too, as presented in Figure 3B. The adsorbed masses of BSM onto microtiter wells in this study, e.g. ca. 645 ng/cm² from 2 mg/mL, are much larger than a previous study, where a saturated adsorbed mass of BSM onto PS particles is reported to be ca. 220 ng/cm² from ca. 22 mg/mL [5], yet much smaller than the adsorbed mass onto another hydrophobic surface, namely polyethylene film ([22], > 2 µg/cm² from 0.45 mg/mL). Discrepancy in the adsorbed mass of BSM onto hydrophobic surfaces across studies can be due to the differences in many experimental parameters, including detection techniques, detailed substrate surface chemistry, exposure time of the substrates to BSM solutions, and the purity of BSM. Nevertheless, it is consistent with the previous studies [5,23] that the adsorption of BSM molecules onto hydrophobic substrates from the aqueous solution with the concentration range employed in this study, maximum up to 2 mg/mL, does not induce a saturated layer, and thus conformational changes of BSM on surface can be occurring within the concentration range selected.

Assuming hexagonal close packing of BSM molecules on surface, the distance between BSM molecules on surface, \( L \), can be calculated as a function of bulk concentration [24], and the results are shown in Figure 4.
Figure 4. The plots of hydrodynamic diameter ($D_h$) of BSM in bulk solution and the distance ($L$) between BSM molecules on surface as a function of bulk solution concentration.

With the increasing adsorbed masses of BSM molecules on surface by increasing the concentration of bulk solution from $10^{-4}$ mg/mL to 2 mg/mL, the distance between neighboring BSM molecules on surface correspondingly decreased from ca. 83 nm to ca. 24 nm. It is of interest to compare these values with the hydrodynamic diameters (Figure 1), as the two values represent the distance between the BSM molecules in bulk solution ($D_h$) and on surface ($L$), respectively. An overlay of the series of $D_h$ and $L$ in Figure 4 shows that given a bulk concentration of BSM, both values are fairly comparable until the bulk concentration is reached to its maximum (2 mg/mL) (Please note that the comparison at 0.01 $\square$ 0.015 mg/mL is unclear due to uncertain $D_h$ of BSM in bulk solution, grey symbols in Figure 4). Even though the estimation of both $D_h$ and $L$ is based on simplistic spherical model of BSM molecules, and thus the shape of molecules is likely to be unrealistic, Figure 4 suggests that the absence of aggregation between BSM molecules displayed in bulk solution (Figure 4) is basically sustained even on surface over a large range of concentration (up to 1 mg/mL). On the other hand, a clearly smaller $L$ value for the BSM adsorbed from 2 mg/mL compared to $D_h$ in the corresponding bulk solution (Figure 4) suggests that some drastic conformational change of BSM molecules may occur on surface at that concentration.
The absence of aggregation between BSM molecules in the concentration range selected in this study is basically based on the repulsive interaction between them. It is tempting to attribute this feature to polyanionic characteristics of BSM and electrostatic repulsion in neutral pH condition. However, a previous surface force apparatus (SFA) study of the interactive forces between two mica surfaces covered with BSM showed that the predominant origin of repulsive interaction between opposing BSM layers is steric rather than electrostatic repulsion, due to bulky structure of BSM and short Debye length in aqueous solution with isotonic salinity as in this study [25]. We propose that a similar interaction mechanism is active between neighboring BSM molecules adsorbed on the surface too.

3.3 Detection of C-terminal regions of BSM on surfaces: EIA

A monoclonal antibody specific for C-terminal amino acids, namely 7461-7474 of Mouse MUC19 antibody, was used to detect the “naked”, unglycosylated end-part of BSM. It has previously been reported that mRNA of BSM is similar to the sequence of mouse MUC19 [26]. Further, according to BLAST analysis, 92% of mouse MUC19 amino acids 7461-7474 have a positive match with bovine MUC19 amino acids. Bovine MUC19 corresponds to BSM [27]. With this information as a starting point, anti-MUC19 EIA plate assay was used to detect the C-termini of BSM molecules adsorbed on the well surface. The results are shown in Figure 5.
Figure 5. EIA signals of BSM bound to hydrophobic surface using MUC19 antibody as a function of BSM surface concentration as detected with BCA method. The presented numbers are average of five experiments; error bars represent standard deviation SD. The numbers are normalized to PBS control = 0 µg/well.

EIA signals from this assay are expected when unglycosylated terminal regions of BSM are exposing and available on surface. Even though the C- and N-terminal groups participate in the interaction with the PDMS surface, they should be readily approachable by other incoming molecules too, such as anti-MUC19 in this study, especially at low surface coverage. As was addressed in the previous section, if BSM molecules retain its conformation, EIA signals should be proportionally increasing with increasing coverage of BSM on the surface. Indeed, the EIA signal initially increased in a linear fashion as the concentration of BSM increased from 0 to ca. 173 ng/cm\(^2\) on the well surface. With further increase in the concentration though, the anti-MUC19 EIA signal was found to be saturated from ca. 173 ng/cm\(^2\) to 300 ng/cm\(^2\). Moreover, the EIA signal even decreased in the intensity at a higher surface concentration, ca. 645 ng/cm\(^2\) BSM on surface. In other words, the availability of C-termini of BSMs to interact with the antibodies does not gradually increase despite a continuous increase of BSM in this surface concentration range. We propose that at the first breakpoint, i.e. the surface coverage of ca. 173 ng/cm\(^2\) resulting from 0.0125 mg/mL in bulk solution, intermolecular interaction between adjacent BSM molecules on surface starts to occur via hydrophobic interaction between C- and N-terminal domains. Unlike in bulk solution,
where hydrophobic patches are shielded from water, hydrophobic terminal domains can be readily exposed in the process of surface adsorption onto hydrophobic substrates. Overlay between the unglycosylated domains may expose the similar number of available C-termini at the outermost surface. A second breakpoint, a slight decrease of EIA signal, is observed at the transition from 1 mg/mL to 2 mg/mL, corresponding to the surface coverage from ca. 300 ng/cm² to 645 ng/cm² on surface. It is noted that at the highest concentration (2 mg/mL), the distance between BSM molecules on surface, $L$, is clearly shorter than the distance between them in bulk solution, $D_h$ (Figure 4), which suggests that a significant compaction of BSM should occur on surface. As unglycosylated C- and N-terminal domains comprise a small section of the entire BSM molecules, and as they form an intimate contact with hydrophobic substrate, much larger, glycosylated domains have a higher propensity to be compacted to accommodate increasing BSM molecules on surface. This, effectively, leads to further shielding of C- and N-terminal domains from water by large glycosylated regions in high surface concentration regime and may explain the decreased anti-MUC19 EIA signal.

3.4 Detection of glycosylated regions of BSM on surface: ELLA

ELLA technique can provide a potential accessibility and conformational changes for glycan moieties on BSM. While MUC19 interacts exclusively with the C-terminus of BSM, the lectins are expected to interact with central, glycosylated PTS region of BSM only. Two lectins, PNA and WGA, were chosen based on different charge and binding affinity to different glycans. PNA is specifically interacting with disaccharide beta-galactosyl-(1,3)-N-acetylgalactosamine (Galβ (1→3)GalNAc) and N-acetyllactosamine [28,29]. Purified PNA comprises of three isoforms with isoelectric points between pH 5 and 7 [30], which gives the lectin an overall slightly negative charge at pH 7.4. WGA is an alkaline lectin that has isoelectric point at ca. pH 8.7 [31] which gives
it positive charge at neutral pH. WGA binds preferentially to N-acetylglucosamine [32], N-acetylenuraminic acid [33], and many of its derivatives [34]. BSM is known to be rich in WGA-preferred glycans, sialic acid in particular, as well as PNA-interacting glycans, beta-galactosyl-(1,3)-N-acetylgalactosamine [35-37]. The combination of PNA and WGA as probing lectins has been employed to detect mucins from human colon tissue extracts [38].

As with EIA, ELLA signal was measured as a function of BSM concentration on the well surfaces, and the signal changes were interpreted to reflect the changes in availability or accessibility of lectin-binding sites (Figure 6).

Figure 6. ELLA signals of BSM bound to hydrophobic surface using wheat germ agglutinin (WGA; squares) and peanut agglutinin (PNA; circles) as a function of BSM surface concentration as detected with BCA method. The presented numbers are average of six experiments; error bars represent standard deviation (SD).

With increasing surface concentration of BSM, the signals from PNA showed a gradual increase in a linear fashion for the entire range tested, i.e. from ca. 55 ng/cm² to 650 ng/cm². Meanwhile, the signal of WGA showed much higher intensity than PNA in low concentration regime. Then, it showed a slight linear decrease between ca.55 and 170 ng/cm² of BSM on the surface, followed by a plateau until ca. 300 ng/cm². Finally, at a higher surface coverage (ca. 645 ng/cm²), a slight drop in the signal was observed (Fig. 6).
Weaker signals from PNA compared to WGA are consistent with the trend reported in a previous ELLA study with both lectins [38]. This is thought to be related to both the weaker sensitivity and low number of corresponding glycans for PNA in BSM. On the other hand, the monovalent binding mechanism of PNA to Galβ (1→3)GalNAc or N-acetyllactosamine makes it a straightforward probe to detect BSM as the pattern of signal increase with increasing surface coverage is fairly linear. This linearity suggests that exposure of the epitopes (glycans) is independent from potential conformational changes of BSM with varying concentration on surface. This may be related to the location of PNA-interacting glycans, such as Galβ (1→3)GalNAc or N-acetyllactosamine, on the BSM molecule; if these sugars are predominantly located at the terminals of the glycan chains, accessibility of PNA to them will be likely to be uninfluenced by the conformational changes.

WGA’s high signal from the low concentration is relating from its own high sensitivity, in the first place. Higher signals obtained from WGA compared to other lectins are consistently reported from other ELLA assays [38,39]. Another reason for the higher signal from WGA ligand in this study is the abundance of WGA-binding glycan, notably sialic acids, in BSM. According to the manufacturer, the amount of bound sialic acid for BSM is 9 17% (BSM Type I-S, SigmaAldrich). An initial decrease, followed by a saturation of the ELLA signal for WGA could be related to the shielding of the corresponding glycans by themselves with increasing concentration on surface. At low surface coverage, BSM is likely to be bound onto surface in a flat-lying fashion that the access of WGA to their binding partners in the glycosylated mid-part of BSM is most feasible. With increasing surface coverage of BSM, the central parts would pack closer and may block each other against the access of incoming molecules. Furthermore, as the target glycans become crowded and the distance between them gets shorter, the possibility of WGA to link with multiple glycans via its well-known multivalency [40,41] become higher with increasing
concentration of BSM on surface; it is known that single WGA molecule can bind with up to eight glycans for its multivalency, and less number of WGA is necessary as the distance between WGA-binding glycans becomes shorter [42]. This scenario is consistent with the conformational changes suggested based on the EIA assay with anti-MUC19 in the previous section; at the highest bulk and surface concentration, BSM molecules become substantially compacted mainly in the glycosylated central part, and thus WGA-binding glycans can become closer and can explain a slight drop in the signal intensity.

3.5 Conformation of BSM on surface as deduced from this study

The EIA and ELLA assay-based probes of BSM molecules on the surface in this study basically support the so-called amphiphilic “triblock copolymer” model of BSM adsorption onto hydrophobic surface from aqueous solution; unglycosylated C- and N-termini act as anchoring units onto the surface via hydrophobic interaction, whereas central, glycosylated regions stretch out to interact with water. Despite Langmuir-isotherm style adsorption of BSM as judged from the mass detection with an optical method [5], however, mucins can undergo complicated conformational changes with varying concentration due to the large, yet flexible structure, as well as the presence of a variety of distinct chemical domains. Using the probe for C-terminus of BSM, the overlay between them from a threshold surface concentration was clearly identified in this study. Two different types of lectins with different selectivity and sensitivity on the central, glycosylated domains of BSM also confirmed the compaction and shielding of underlying C- and N-terminal domains with increasing surface coverage.

4. Conclusions
In this study, we have demonstrated that biomolecular assays that are traditionally used to detect the presence/absence of certain epitopes, such as EIA and ELLA, can be exploited to investigate the conformational changes of BSM molecules with varying concentration on surface. This idea was inspired from that the large and flexible structure of BSM may display a complex conformational change with increasing surface coverage, and the two groups of probes, antibody and lectins, that are specifically sensitive to glycosylated and nonglycosylated regions of BSM can provide the information on the conformational changes of respective domains. DLS and CD spectroscopy results firstly indicated that no noticeable aggregation of BSM is occurring up to 2 mg/mL in bulk solution. BCA assay showed that the adsorbed mass is proportionally increasing to the bulk concentration of BSM. EIA and ELLA assays were also consistent with the conventional amphiphilic, triblock copolymer model for the adsorption of BSM onto hydrophobic surface; hydrophobic C- and N-terminal domains act as anchoring groups, whereas central, glycosylated domains are stretched to bulk water for the hydrophilicity. Nevertheless, EIA and ELLA assays further indicated that delicate conformational changes are accompanied in the surface adsorption process. It is most notable that C- and N-terminal domains of surface-adsorbed BSM molecules start to be shielded from a threshold surface concentration of BSM, due to increasing compaction of larger and flexible glycosylated domains.

5. Acknowledgements

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References

[24] The distance between BSM molecules was calculated according to the equation, $L = \left(\frac{6}{3x/2}\right)^{1/2}$ where $x$ is the number of BSM molecules per unit area (nm$^2$). The molecular weight of BSM was assumed to be 2 MDa.


Appendix 3: Feasibility of bovine submaxillary mucin (BSM) films as biomimetic coating for polymeric biomaterials

BSM has previously been proposed as a biocompatible surface coating for medical devices. In this study, we were interested in the anti-fouling, adsorption and tribological properties of BSM adsorbed onto polystyrene and polydimethylsiloxane surfaces.

My contributions to this study were OWLS surface adsorption measurements and testing the wettability of the surface substrates.
FEASIBILITY OF BOVINE SUBMAXILLARY MUCIN (BSM) FILMS AS BIOMIMETIC COATING FOR POLYMERIC BIOMATERIALS

Seunghwan Lee*, Jan Busk Madsen, Kirsi I. Pakkanen
Department of Mechanical Engineering
Technical University of Denmark
DK-2800 Kgs. Lyngby, Denmark
*seele@mek.dtu.dk

ABSTRACT
Feasibility of bovine submaxillary mucin (BSM) films generated via spontaneous adsorption from aqueous solutions onto polydimethylsiloxane (PDMS) and polystyrene (PS) surfaces have been investigated as biomimetic coatings for polymeric biomaterials. Two attributes as biomedical coatings, namely anti-fouling properties and lubricity, have been focused on in this study. Optical waveguide light-mode spectroscopy (OWLS) and fluorescence microscopy studies have shown that albumin, fibrinogen, immunoglobulin G, and serum rapidly adsorb onto BSM layers formed on PDMS and PS surfaces. Pin-on-disk tribometry, employing compliant PDMS as tribopairs, has shown that BSM coatings generated on PDMS surface via spontaneous adsorption from aqueous solution has effective lubricating properties, but for very limited duration only.

KEY WORDS
Bovine submaxillary mucin (BSM), biomedical coating, lubricity, biofouling

1. Introduction
Recently, mucin has been proposed as potent biomedical coatings [1-5]. This idea is based upon an assumption that since mucin/mucus represent outmost layer of epithelium, biomaterials coated with mucins may be recognized as “self” rather than “foreign” bodies by the human immune system. In fact, mucin films generated on the surfaces of synthetic materials have shown improved hydrophilicity [6], resistance to the formation of biofilms (i.e. suppression of bacterial growth) [5,7,8], and favorable immune cell responses [1,3,4], which collectively support the viability of mucin coatings for implants. In particular, the studies on immune cell responses [1,3,4] were carried out either by employing mucin coatings alone [1] or composite films with various proteins [3,4], and a superior effect from composite film is reported [3,4]; in fact, involvement of proteins is expected to provide more realistic assessment of biocompatibility of mucin coatings for implants, since the adsorption of proteins onto implant surfaces, especially those associated with inflammation and coagulation, is unavoidable on the time scale of implant use, and the interaction with immune cells occurs after the formation of protein layers [9,10].

In this work, we address the question of whether mucin coatings have a potential as anti-fouling films as well, i.e. the capabilities to suppress non-specific adsorption of proteins. As mentioned above, in a long-term application of biomaterials, such as implantation in human body as prosthesis, formation of protein layers on surface is not only unavoidable but also rather necessary to regulate favorable immune response. On the contrary, it is generally required to possess anti-fouling properties for biomaterials to be used with short-term contacts with biofluids, such as biosensors and biomedical devices, for their optimal operations [11,12]. Presently, synthetic polymer coatings, e.g. oligo- and polysaccharides [13,14], polyacrylates [15], and poly(ethylene oxide) (PEO) [16-18], have extensively been studied as anti-fouling coatings for biomaterials.

To date, a majority of the studies of mucin coatings for biomaterials has been carried out targeting hydrophobic, polymeric surfaces [1-8]. On polymeric surfaces, mucins adsorb spontaneously from aqueous solution due to its amphiphilic characteristics [1-5]. A simplistic view for this adsorption is based on that unglycosylated N- and C-termini act as anchoring groups for their hydrophobicity, whereas glycosylated regions stretch out to interact with bulk water, as schematically illustrated in Figure 1.

![Figure 1. A schematic of (a) mucins and (b) proposed conformation at water/solid interface.](image-url)

The main objective of this work is to examine the feasibility of mucins as anti-fouling coatings for polymeric biomaterials. Feasibility of mucins as non-fouling coatings is firstly found from its capabilities to suppress bacterial growth on polymeric surfaces [5,7,8]. Previous studies on immune responses of BSM coatings...
including exposure to proteins have shown facile adsorption of proteins onto mucin coatings [3,4], but characterize the tribological properties of mucin coatings for polymeric biomaterials. Once used as biomedical coatings, mucins are additionally attractive for its excellent lubricity, which can be beneficial for various tissue-contact devices, such as stents, catheter, and endoscopes. In addition to its genuine lubricating properties at mucosal lines, previous studies have shown excellent lubricating properties of mucins at the surfaces composed of synthetic materials [19-22]. It is cautioned though that the experimental conditions of those studies involved either mucin solutions, for which excess mucin molecules (> 1 mg/ml) can readily replenish potentially worn-out mucin layers [20,21] or extremely mild external load in nanoscopic scale contacts [19,22], both of which are substantially different from a layer of coatings on surface as lubricant.

2. Materials and Methods

2.1 Materials

BSM and purification

Bovine submaxillary mucins were purchased from Sigma-Aldrich (M3895-1G, type I-S, St. Louis, MO), and purified by anion exchange chromatography by modifying the methodology proposed by Caldwell et al. [2]. Briefly, commercial BSM was dissolved in Na-acetate buffer (10 mM Na-acetate, 1 mM EDTA, pH 5.0) at 4 °C to a final concentration of 10 mg/ml, filtered through a 5 µm sterile filter (Pall Corporation, Cornwall, U.K.), and fractionated according to the charge on a High Load 16/26 Q Sepharose High Performance Anion Exchange Column (GE Healthcare Life Sciences, USA) by elution with a high salt concentration Na-acetate buffer (10mM Na-acetate, 1mM EDTA, 1.2M NaCl, pH 5.0). Fractions were analyzed with SDS-PAGE (data not shown). The fractions containing BSM were pooled and dialyzed against MilliQ-grade water and freeze-dried. The “purified” BSM was stored at -20 °C and desiccated prior to use. All steps were performed at 4 °C to minimize the possibility of proteolytic degradation. Since purified BSMs according to this procedure only were used in this work, they are simply denoted as BSM.

Substrates

Poly(dimethylsiloxane) (PDMS) and polystyrene (PS) were employed as substrates for model polymeric biomedical devices and sensors. PDMS were employed for OWLS studies (see section 2.3) and tribological studies (see section 2.5) whereas PS was employed for fluorescence microscopy studies (see the section 2.4). Both PDMS (PDMS disk, see below) and PS (cut from microtiter cover lid, see below) were used for wettability studies. For tribological studies, Sylgard® 184 PDMS kit (Dow Corning) was employed to fabricate PDMS pins and disks in molds. The base and crosslinker were mixed at a 10:1 ratio, and the transferred fluids in molds were cured in an oven (at 70 °C) overnight. For OWLS, ultra-thin layer of PDMS film (ca. 20 nm) was generated on short-term interaction with proteins has rarely been studied. Another interest of this work is to OWLS waveguides by spin-coating. For PS, 96 well microtiter plates (BRAND PureGrade, BrandTech Scientific, Essex, UK) were employed without further treatment.

Proteins

Albumin (from bovine serum), Immunoglobulin G (IgG, from bovine serum), and fibrinogen (from bovine plasma, Type I-S) were purchased from Sigma-Aldrich (St. Louis, MO). Serum (Alpha Calf, non-iron) was purchased from ThermoFisher Scientific. Fluorescein isothiocyanate (FITC)-conjugated albumin (from bovine serum) were purchased from Sigma-Aldrich, and FITC-conjugated fibrinogen was purchased from Cell Sciences Inc. (Canton, MA). The concentrations of protein solutions, within the range of normal physiological concentrations, are shown in Table 1. It is noted that the concentrations for FITC-albumin and FITC-fibrinogen for fluorescence microscopy studies are 10% of those of unlabeled proteins for OWLS studies.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Concentration for OWLS</th>
<th>FITC-conjugated proteins</th>
<th>Concentration for fluorescence microscopy</th>
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<td>Albumin</td>
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<td>FITC-Albumin</td>
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<tr>
<td>Serum</td>
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2.2 Generation of BSM Coatings

BSM coatings were generated by placing a drop (100 µl) of BSM solution on PDMS-coated waveguide chips (for OWLS) or 96 microtiter plate wells (for fluorescence microscopy) for 24 hours at 4 °C. 0.5 mg/ml of BSM in PBS buffer was used as standard throughout this work. This concentration is higher than that used in the studies from literature with similarly purified BSM [1,3,4] (0.25 mg/ml), but is lower than that in another study employing commercial BSM without purification [5] (1 mg/ml). 0.25 mg/ml BSM solution was also used for an OWLS control experiment. For BSM coating of PDMS disks (30 mm in diameter and 5 mm in thickness), 1 ml of BSM solution was placed on the surface, and hemisphere PDMS pins (3 mm in apex diameter) were immersed in 0.5 ml BSM solution for 24 hours at 4 °C. For wettability studies, a drop (100 µl) of the BSM solution was placed on PS surface (a cover slip). PDMS disks were used for wettability studies prior to tribological measurements. After 24 hours of incubation, the surfaces were rinsed.
with PBS buffer, gently dried in ambient, and then used for further studies.

2.3 Optical Waveguide Light-Mode Spectroscopy (OWLS)

OWLS (MicroVacuum Ltd, Budapest, Hungary) is an optical technique that detects the adsorption of macromolecules from solution to solid/liquid interface by probing the change of refractive index at the vicinity of solid surface upon adsorption. The adsorbed mass is deduced from the change of Fejter equation [23]. Further details on the operational principles of OWLS are available in previous publications [14,18].

To provide water/PDMS interface, OWLS sensor chips (Si_{0.25}Ti_{0.75}O_{2}) were coated with PDMS by spin-coating with PDMS. OWLS experiments were carried out for two purposes. Firstly, adsorbed mass of BSM on PDMS surfaces by incubation for 24 hours was quantitatively estimated. For this experiment, a PDMS-coated OWLS sensor chip was exposed to PBS buffer solution in a flow cell until a stable baseline was obtained. Then, BSM solution was injected into the flow cell. The mass uptake was allowed for 24 hours. Finally, the flow cell was rinsed with PBS buffer solution, and adsorbed mass was calculated by assuming dn/dc, refractive index increment, as 0.182. Secondly, PDMS-coated sensor chips that were further coated with BSM (section 2.2) were employed in a flow cell, and exposed to each protein solution to investigate the adsorption behavior of proteins onto BSM coatings. The exposure of BSM coating to protein solutions was up to 1 hour.

2.4 Fluorescence Microscopy

Fluorescence microscopy was carried out to visualize the interaction of PS surfaces, in the presence and absence of further surface modification with BSM coatings, with the proteins. Exposure times were 10 seconds, 1 minute, 10 minutes, 1 hour, and 24 hours, after which the wells were washed 3 times with PBS buffer solution. After the last wash, the wells were emptied and imaged dry immediately after sample preparation. Imaging was performed using an inverted epifluorescence microscope (Axiovert A1, Zeiss, Jena, Germany) with LED illumination. All images within each time point were taken with the same camera settings. Image analysis was performed using ImageJ software [24].

2.5 Pin-on-Disk Tribometry

Pin-on-disk tribometer (CSM, Switzerland) was employed to test the lubricity of BSM coating against tribological stress [18]. In this approach, loaded pin (by dead weight) is forming a contact with rotating disk, and generated friction forces are measured with a strain gauge. To emulate 'soft' tribological contacts involving biological tissues, PDMS was employed as the tribopair, both pin and disk, mainly due to its compliance (Young’s modulus = 2 MPa, and Poisson ration = 0.5). Under the experimental setup employed in this work (1 Newton load applied on 3 mm hemisphere pin on plane), the contact pressure is ca. 0.51 MPa (≈ 5 atm). To evaluate the lubricity of BSM coating on PDMS surfaces, coefficient of friction, μ (= friction force/load) of the sliding contact between BSM-coated PDMS surfaces was obtained over 1,000 rotations (corresponding to ca. 38 m in total sliding distance). For reference, μ values obtained from the sliding contacts between bare PDMS surfaces were also obtained. The sliding speed was set at 10 mm/s. All the measurements were carried out in distilled water at 37 °C.

3. Results

3.1 Adsorption of BSM onto Surfaces

3.1.1 Wettability

Wettability by water provides a first indication on the formation of BSM films on PDMS and PS substrate. Static water contact angles on PDMS-coated PDMS disks and PS plates changed from 107° (PDMS) and 109° (PS) to 56° (PDMS) and 32° (PS), respectively (Figure 2).

These values are somewhat different from those reported in literature [6], possibly due to the differences in BSM purity, concentration, as well as the time, temperature, and methods for BSM incubation. Nevertheless, substantial decrease in static water contact angles on both polymeric surfaces after incubation with BSM solution for 24 hours supports a formation of BSM coatings on the surfaces.

3.1.2 OWLS Studies

More quantitative information on the adsorption behavior of BSM onto PDMS surface was obtained from OWLS studies. Figure 3 shows surface adsorption profiles of BSM from two different BSM solutions (0.25 and 0.5 mg/ml in PBS) onto PDMS surfaces.
The adsorbed masses obtained after 24 hours of adsorption and subsequent rinsing were ca. 119 ng/cm$^2$ and ca. 175 ng/cm$^2$ from the BSM solutions of 0.25 mg/ml and 0.5 mg/ml, respectively. As noted in Figure 3, adsorption of BSM onto PDMS surface from aqueous solution was observed to be extremely slow and a plateau was not reached even after 24 hours for either concentration. Slow adsorption kinetics of BSM onto PDMS surface is thought to be primarily resulting from the barrier of accumulating charged macromolecules onto non-polar surface. As is well known, mucin is negatively charged under neutral pH conditions due to sulfate and carboxylic acid groups present both on the peptide backbone and carbohydrates [25,26], and isoelectric point of BSM is reported to be around 3 [27]. Thus, adsorption process may involve not only arrival of BSM at the surface, but also rearrangement, possibly involving already-adsorbed BSM molecules on the surface.

Whether the generated BSM film obtained according to this procedure is a completed monolayer or not is unclear. Fairly lower adsorbed mass obtained from 0.25 mg/ml BSM solution than 0.5 mg/ml, by ca. 30%, and the lack of plateau even after 24 hour of adsorption (Figure 3) suggest that the coverage of PDMS surface with BSM is likely to be incomplete even for the case of 0.5 mg/ml. However, due to the large size and flexibility, BSM on PDMS surface can possibly cope with a variety of different conformations, and continuous increase of the adsorbed mass shown in Figure 3 may indicate the formation of multiple layers. For further experiments, adsorption of BSM from 0.5 mg/ml for 24 hours was taken as standard.

### 3.3 Interaction of BSM Coatings with Proteins

#### 3.3.1 OWLS Studies

It was of primary interest in this work whether BSM films on hydrophobic surfaces can reveal anti-fouling properties following short-term exposure (less than 1 hour) to proteins. Shown in Figure 4 is a comparison of the adsorption profiles of fibrinogen onto bare PDMS vs. BSM-coated PDMS surfaces as studied by OWLS.

Upon exposure of the surfaces, either bare PDMS or BSM-coated PDMS, to fibrinogen solution, a majority of final adsorbed mass (obtained rinsing after 1 hour) was already obtained within a few seconds, and then a much slower further adsorption process followed. The final adsorbed masses of fibrinogen onto bare PDMS and BSM-coated PDMS surfaces were ca. 295 ng/cm$^2$ and 207 ng/cm$^2$, respectively. Despite the reduction of fibrinogen adsorption by 30%, this behavior is far from effective coatings such as PEO-based films on a PDMS surface that impart excellent non-fouling properties [18].

The same OWLS measurements were extended to other proteins, including albumin, IgG, and serum. The results are shown in Figure 5.
protein adsorption during short-term interaction. This is in contrast to previous studies that show BSM coatings suppress bacterial adsorption [5,7,8].

### 3.3.2 Fluorescence Microscopy Studies
Adsorption behavior of proteins onto BSM-coated, hydrophobic surfaces was studied also by employing PS microtiter plates and fluorescence microscopy. It is noted that while the BSM solution (0.5 mg/ml), incubation time (24 hour), and temperature (4 °C), are identical with the OWLS experiments, the concentrations of FITC-conjugated albumin and fibrinogen are 10% of those for OWLS. Exposure durations of PS and BSM-coated PS surfaces to protein solutions were 10 seconds, 1 minute, 10 minutes, and 1 hour, on different wells on the same plate. An example of microscope images obtained from the case of albumin and fibrinogen by exposing for 10 seconds is shown in Figure 6.

![Figure 6. Fluorescence microscopic images of FITC-conjugated albumin and FITC-conjugated fibrinogen adsorbed onto bare PS and BSM-coated PS surfaces (exposure of proteins for 10 seconds). The dimension of the image is 667 μm x 470 μm.](image)

Despite 10 times lower concentration compared to OWLS experiments, exposure of both bare PS and BSM-coated PS surfaces to two types of proteins for only 10 seconds led to a significant increase in fluorescence intensity, confirming that the adsorption of albumin and fibrinogen onto BSM-coated and bare PS surfaces is very rapid. Although the fluorescence microscopic images shown in Figure 6 were obtained from individual microtiter wells, both hardware and software settings of the fluorescence microscope were identical for each image that the relative comparison in fluorescent intensity is valid. Thus, semi-quantitative analysis of the fluorescence intensity (average over lateral central line from each image) has been plotted in Figure 7 (a). For comparison, the relative fluorescence intensities obtained from the images obtained by exposing the surfaces to the protein solutions for 1 hour are also shown in Figure 7 (b). It is noted that while the relative fluorescence intensities for the same protein solution exposure time is valid, the comparison between the intensities with different exposure time is not for different camera parameter settings.

![Figure 7. Relative fluorescence intensities from the images obtained after exposure of the surfaces to the protein solutions for (a) 10 seconds (b) 1 hour.](image)

Both data obtained from 10 seconds and 1 hour support that BSM coatings formed on PS surfaces do not provide effective anti-fouling properties against adsorption of proteins.

### 3.4 Tribological Properties of BSM Coatings
The results shown in Figure 8 address the pin-on-disk tribometry experiments carried out by involving PDMS pin and disk, with or without BSM coatings at 37 °C.

![Figure 8. Coefficient of friction (μ) plotted as a function of rotation measured by pin-on-disk tribometer between PDMS surfaces in distilled water, with or without BSM coatings. The load was 1 N, speed 10 mm/s, and the 1,000 rotations correspond to 38 m in total sliding distance.](image)

Even though initially very low frictional properties are observed (μ close to 0) for the case of BSM-coated PDMS, the μ values gradually increased in the course of 1,000 rotations to a level of bare PDMS substrate in the end. This observation suggests that BSM coating is gradually removed from the surface during the test.
4. Discussion
The results obtained in this work mostly point to insufficient feasibility of mucin coatings for polymeric biomaterials, in terms of both anti-fouling properties and lubricity. However, it is not clear from this work whether it is resulting from genuine properties of BSM or related to the preparation of mucin coatings adopted in this work. While spontaneous adsorption from aqueous solution is certainly attractive from a manufacturing point of view due to its simplicity, two main problems are encountered.

Firstly, the stability of mucin films is questionable since no specific chemical bonding is involved to stabilize the anchoring of mucins on the surface. This problem is most obvious from the tribological properties, where excellent lubricating properties are shown, but only for a very limited duration or sliding distance. Weak binding of BSM coatings onto substrate may influence on anti-fouling properties as well; weakly bound mucins may allow for replacement with incoming proteins, even partially, more easily than strongly bound mucins. Convoluted to this problem is the possibility of incomplete coverage of the surface with BSM coatings under the experimental condition chosen in this work (24 hour of incubation with 0.5 mg/ml BSM solution). Secondly, due to spontaneous adsorption, there is no control on locating chemical groups to the outmost layer. Even if the schematic shown in Figure 1 reflects the reality of the conformation of mucins on the surface, various chemical groups can be exposed to the topmost layer. For example, both unglycosylated regions and glycosylated regions can be exposed in “flat-lying” or “train” conformation, whereas unglycosylated regions can be better shielded if glycosylated regions take “loop” conformation. Furthermore, unglycosylated regions can also be exposed if charged amino acids are included in the regions. Continuous increase of the adsorbed mass of BSM onto PDMS surfaces even after 24 hours of adsorption from 0.5 mg/ml solution (Figure 3) may reflect the continuous rearrangement of the conformation of BSM molecules to adopt more BSM molecules on the surface. To clarify both issues, it is necessary to perform the experiments with higher amounts of BSM coatings on the surface by employing longer incubation and/or higher concentration of BSM solution in the future. Furthermore, the coating strategy can be drastically changed from self-assembly via spontaneous adsorption to directed assembly to impart more controlled conformation of mucin molecules on the surface. For instance, a previous study on bacterial adhesion by BSM coating [5] reported that the efficacy of suppressing bacterial growth was fairly different depending on whether bare PS substrates are used or they are pre-coated with PMMA-b-PAA, a mucoadhesive film.

Insufficient anti-fouling properties of BSM coatings shown in this work is in strong contrast to the reported efficacy of BSM coatings to suppress bacterial adsorption [5,7,8]. While the detailed adsorption mechanisms of bacteria vs. proteins onto mucin layers must be clarified, we speculate that this difference is firstly related to the difference in sizes of bacteria and proteins. For example, a tiny “vacant” area can be an effective docking site to facilitate the adsorption for relatively small proteins, but not necessarily for much larger bacteria. Again, experiments with much denser BSM coatings can provide useful information on this issue too.

As mentioned above, the reason for the very limited duration of revealing excellent lubricity by the mucin coatings (Figure 8) is clearly due to weak binding onto the surface. When the same experiments were performed by employing BSM solution (1 mg/ml), as opposed to the coating, extremely low coefficient of friction, on the level of \( \mu = 0.01 \sim 0.04 \) is maintained for the same length of sliding distance (data not shown). This is because even if the initially formed BSM coating in solution is rubbed away from tribostress, excess amount of BSM molecules in solution can quickly replace the worn area, and thus, lubricating efficacy can be maintained for much extended duration. While this approach is readily applicable to lubrication of bearings, it is not straightforward for biomedical applications, such as endoscopy, catheter, and stents. Similarly with the non-fouling properties, it is necessary to strengthen the binding to graft mucins onto surface for improved tribological properties.

5. Conclusion
In this work, we have generated BSM films on PDMS and PS surfaces via spontaneous adsorption from aqueous solutions for feasibility tests of them as non-fouling and lubricious coatings for polymeric biomaterials. The conclusions can be drawn as follows.

1. Adsorption of BSM onto PDMS surfaces from aqueous solution was observed to be extremely slow process. While 175 ng/cm\(^2\) was obtained from the exposure of PDMS surface to 0.5 mg/ml BSM solution for 24 hours, it appears that higher mass can be adsorbed by employing longer exposure and/or higher concentration of BSM solution.

2. BSM coatings obtained from spontaneous adsorption onto hydrophobic surfaces did not show sufficient efficacy in supressing adsorption of proteins, including albumin, IgG, fibrinogen, and serum, especially compared to synthetic polymer coatings. However, its origin is not clear at this stage; apart from a possibility of intrinsic properties of BSM layers in interaction with proteins, coverage, binding strength, and exposed specific chemical groups of BSM films achieved according to the method in this work may also be related.

3. BSM coatings on PDMS surfaces have shown an excellent feasibility of lubricating soft contacts, but its efficacy is very limited in duration. This is attributed to weak binding strength of BSM onto PDMS surfaces achieved according to the method in this work.
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References
Appendix 4: Unique Non-Fouling Surface (NFS) Generated with a Commercial Bovine Submaxillary Mucin Films: Resistance to Nonspecific Adsorption of Proteins

In this study, we were interested in investigating the non-fouling properties of BSM in regards to proteins adsorbing onto mucin coated surfaces. Comparisons were made between two different types of arBSMs, purified BSM and BSM mixed with BSA in a ratio of 2:1.

My contributions to the work in this study were purification of BSM, SDS-PAGE analysis and OWLS surface adsorption measurements.
Unique Non-Fouling Surface (NFS) Generated with a Commercial Bovine Submaxillary Mucin Films: Resistance to Nonspecific Adsorption of Proteins

1. Introduction

Commercially available mucins are known to contain various non-mucinous biomolecules.\textsuperscript{1,2} They could be some integral components of mucus gels, such as lysozyme, immunoglobulins, lactoferrin, and salts. But, some of them could have been introduced in the purification steps by the manufacturer, which is likely to include, in particular for bovine submaxillary mucins (BSM), gland homogenization. Thus, commercially available mucins do not necessarily represent pure mucin molecules, and non-mucinous molecules in them may complicate their biophysical properties, especially when they are exposed to the interaction with other entities.

In this study, we report that biomolecular films generated from a particular type of commercially available mucin on hydrophobic surface reveal excellent resistance against non-specific adsorption of proteins. Past studies have often employed mucin films to construct non-fouling surfaces (NSF),\textsuperscript{3-8} but they have mostly aimed to suppress the adhesion of cells,\textsuperscript{3-5} bacteria,\textsuperscript{6} or platelet,\textsuperscript{7} whereas the resistance towards proteins by mucin films is rarely reported to date. In fact, rather strong interactions of proteins onto mucin films has been reported.\textsuperscript{2,4} Moreover, protein resistance in the present study was observed from only one particular type of commercial BSM, namely “BSM type I” from Sigma Aldrich, but not from other types, such as “BSM type I-S” or highly purified BSM. Due to the presence of high amount of non-mucinous molecules, commercially available mucins can be considered as a “composite” of mucin and these biomolecules. The results in this study suggest that the combination of BSM and other biomolecules can show synergetic effect to display resistance towards non-specific adsorption of proteins.
2. Materials and Methods

2.1 Biomolecules

Two types of commercial bovine submaxillary mucins were purchased and used as received in this study; (1) BSM type I (M-4503, bound sialic acid content ~5%) and BSM type I-S (M3895-1G, type I-S, bound sialic acid 9 to 17%, both from Sigma-Aldrich). They are abbreviated as “BSM(I)” and “BSM(I-S)” in the following. BSM(I-S) was further purified to remove impurities by means of one step anion exchange chromatography. Details on the purification procedure are provided in a previous publication. The purified BSM by means of anion exchange chromatography is abbreviated as “aeBSM”. Bovine serum albumin (BSA) was also purchased from Sigma-Aldrich (Brøndby, Denmark). FITC-conjugated fibrinogen was purchased from Cell Sciences Inc. (Canton, MA). Serum (Alpha Calf, non-iron) was purchased from ThermoFisher Scientific. The concentration for FTTC-fibrinogen for fluorescence microscopy studies was 0.3 mg/mL and of those of unlabeled proteins for OWLS studies. PBS was employed as neutral buffer throughout the study.

2.2 Substrates

Poly(dimethylsiloxane) (PDMS) was employed as substrates. For OWLS experiments, ultrathin PDMS layer was prepared by spin-coating onto waveguide (Si$_{0.25}$Ti$_{0.75}$O$_2$) with the thickness of 15-20 nm, whereas thin PDMS slabs with the thickness of ca. 0.5-1 mm were used for fluorescence microscopy experiments. Both PDMS were fabricated with two-component Sylgard® 184 PDMS kit (Dow Corning, Midland, MI). For the ultrathin films on OWLS waveguides, the base and crosslinker were mixed at the ratio of 10:3. The mixture was then dissolved in hexane at the concentration of 0.5%, followed by spin-coating on the OWLS waveguide chips at 2 000 rpm for 60 sec. For the thin slabs for microscopy, the mixed fluids of base and crosslinker were poured onto a
silicon wafer (6 inch in diameter). Both of them were cured in an oven (at 70 °C) overnight. The thin slabs were peeled off from the silicon wafer and placed on microscope glass for further experiments.

2.3 Generation of BSM films and protein resistance tests

Generation of BSM films and proteins resistance tests were carried out in two ways. Firstly, both the generation of BSM coating and protein resistance tests were conducted in an OWLS flow cell. In this approach, the PDMS-coated OWLS waveguide surface was exposed to PBS buffer solution until a stable base line was established first. Then, the surface was exposed to BSM solution to generate BSM film via spontaneous adsorption of BSM molecules onto PDMS surface. After the exposure for varying duration (30 min to 24 hr), the film was rinsed with buffer, leaving the strongly bound molecules only. The formation of BSM film was completed by quantification of the adsorbed mass. Then, BSM film was exposed to target protein solution, e.g. serum, for 10 min. After rinsing the flow-cell with buffer again, the adsorbed mass of proteins was quantified. The protein resistance was assessed by comparing the adsorbed protein mass on the BSM-coated PDMS vs. bare PDMS surface. In the entire procedure, the PDMS substrate remained in aqueous environment. Secondly, BSM films were formed on PDMS slab by placing a drop (ca. 100 µl) of BSM solution on PDMS slab for 24 hours at 4 °C. The substrate was rinsed with PBS buffer solution, and gently dried in ambient. Then, the BSM film generated on PDMS slab was exposed to fluorescence-labeled fibrinogen. After rinsing, the fluorescence intensity was measured by microscopy. The protein resistance properties were quantified by comparing the fluorescence intensity on the BSM-coated vs bare PDMS slabs. For both approaches, the concentration of BSM solution was 1 mg/mL, unless otherwise mentioned.
2.4 Optical Waveguide Light-mode Spectroscopy (OWLS)

OWLS (MicroVacuum Ltd, Budapest, Hungary) is an optical technique that detects the adsorption of macromolecules from solution to solid/liquid interface by probing the change of refractive index at the vicinity of solid surface upon adsorption. The adsorbed mass is deduced from the de Feijter equation. For all the biomolecules, the $dn/dc$ values were assumed to be 0.182 cm$^3$/g. Further details on the operational principles of OWLS are available in previous publications.

2.5 Fluorescence microscopy

Fluorescence microscopy was employed as an alternative means to characterize the protein resistance properties of BSM films. For this study, FITC-tagged fibrinogen was employed as a probe protein, partly to complement serum proteins. Exposure times varied from 10 seconds, 10 minutes, and 1 hour, after which the substrates were rinsed 3 times with PBS buffer solution. Then, the sample substrates were dry and imaged immediately. Imaging was performed using an inverted epifluorescence microscope (Axiovert A1, Zeiss, Jena, Germany) with LED illumination. All images within each time point were taken with the same camera settings. Image analysis was performed using ImageJ software. As the fluorescence intensity was not calibrated to adsorbed mass of fibrinogen, the relative comparison is valid for the data obtained from the same setting and in the same figures.

2.6 Circular dichroism (CD) spectroscopy

Far UV circular dichroism (CD) spectra of various protein solutions (1 mg/mL) were acquired with a Chirascan spectrophotometer (Applied Photophysics Ltd., Surrey, UK) at room temperature (ca. 22 °C). A cylindrical quartz cuvette with 10 mm path length (Hellma GmbH & Co. KG, Müllheim, Germany) was used. The wavelength range was selected from 280 to 190 nm with step
size of 2 nm and bandwidth of 1 nm. The far-UV CD signals of the buffer background were subtracted from the data. The presented data are average of three independent measurements, each averaged of three scans.

3. Results

3.1. OWLS: suppression of serum adsorption

A representative mass adsorption profile to form BSM(I) films on PDMS surface, followed by exposure to serum, as characterized by OWLS, is presented in Figure 1. The time “0” is defined as the point where BSM(I) solution is injected into the flow cell after the stabilization of baseline. The points of “a”, “b”, and “c” represent the points of “rinsing BSM(I) solution with buffer”, “injection of serum”, and “rinsing serum with buffer”, respectively.

![Figure 1](image)

Figure 1. A representative mass adsorption profile of BSM(I) (1 mg/mL) for the formation of BSM films files: a = rinsing of BSM(I) solution with buffer, b = injection of serum, c = rinsing of serum with buffer.

The adsorbed mass of BSM(I) for the particular experiment in Figure 1(a) was ca. 135 ng/cm² after the exposure for 30 min. Near-perfect rejection of adsorbing serum proteins (close to 0 ng/cm² after rinsing with buffer, “c”) shown by the BSM(I) films. The films generated with BSM(I-S) or
aeBSM also showed reduction in the adsorbed mass of serum compared to bare PDMS surface, yet not as effectively as BSM(I), by leaving the adsorbed mass of ca. 30 to 50 ng/cm² (data not shown). As all types of BSMs showed a slow, yet gradual increase in the adsorbed mass within the first a half as shown in Figure 1, it can be argued that the completion of BSM film formation may take much longer and different duration for different types of BSMs. For this reason, the exposure time of PDMS surface to BSM solution was extended to 24 hrs in order to generate thicker BSM films and the same protein resistance tests were conducted. A representative result for aeBSM is shown in Figure 2.

![Mass adsorption profile of aeBSM (1 mg/mL) for 24 hours, followed by exposure to serum. Inset is a magnified plot for the serum injection and rinsing, and the arrow indicates the amount of adsorbed serum on the aeBSM film (ca. 60 ng/cm²).](image)

As a result of the extended formation time of aeBSM film, the adsorbed mass was increased to ca. 370 ng/cm². Nevertheless, inferior resistance of aeBSM film against adsorption of serum remained unchanged by revealing subsequent adsorption of serum by ca. 60 ng/cm². BSM(I) films also showed increased adsorbed mass after extended adsorption time, but generally 30 min to 1 hr was sufficient to generate near-perfect protein-resistant films. Average values and standard
deviations from multiple measurements (3 to 4) for the adsorbed serum protein amount are presented in Figure 3 for all three types of BSM films (24 hr exposure as standard).

![Figure 3](image_url)

Figure 3. OWLS data for the formation of BSM films with BSM(I-S) by exposing for 24 hours, and subsequent exposure to serum on PDMS surface. The concentration of BSM solutions were 1 mg/mL.

3.2. **Fluorescence microscopy: suppression of fibrinogen adsorption**

The resistance of BSM FITC-tagged fibrinogen was used as probe protein. As mentioned in the experimental part, fluorescence microscopy experiments included the exposure of the BSM films to ambient in the preparation procedure, which is an unavoidable step if the mucin coating is further developed as a part of manufacturing process. In order to investigate the influence of incubation time of BSM films for protein resistance, the incubation time was varied, 10 sec, 10 min, and 1 hr incubation. No particular difference was observed, and presented in Figure 4 are the results obtained from 10 min exposure of FITC-tagged fibrinogen to BSM films.

![Figure 4](image_url)

Figure 4. Intensity of FITC-tagged fibrinogen onto different BSM films.
While all types of BSM films exhibited reduction in adsorbed amount of fibrinogen (fluorescence intensity) compared to bare PDMS surface, the efficacy of suppression is clearly superior for BSM(I) films.

3.3. Compositional and structural analysis of BSMs

The difference between three types of BSM samples, BSM (I), BSM (I-S), and aeBMS, was studied by means of SDS-PAGE and CD spectroscopy. The SDS-PAGE results are shown in Figure 5.

![SDS-PAGE Image]

Figure 5. SDS-PAGE with CBB stain for (1) BSM(I-S), (2) BSM(I), (3) aeBSM, and (4) the mixture of aeBSM:BSA samples.

The proteins in the top part of the lanes with the molecular weight ca. 200 kD are verified to be BSM or BSM fragments, as confirmed from PAS staining.² It is apparent that BSM(I) contains various small protein species (m.w. < 200 kDa), most of which are absent in other types of BSMs. BSM(I-S), despite being another type of commercial BSM from the same manufacturer, is essentially purer form of BSM. BSA was identified as a major non-mucinous protein component in BSM(I-S), as is also shown in Figure 5.² aeBSM is distinguished from BSM(I-S) in that even BSA
is essentially removed as a result of additional purification step. It is noted that that another lane of SDS-PAGE data displays the data for aeBSM:BSA at the ratio of 2:1. This will be discussed below.

Far UV CD spectroscopy was employed to investigate the secondary structure of different BSM molecules. The results are shown in Figure 6.

Figure 6. Far UV CD spectra obtained from various BSM solutions, BSA, and the mixed solution of aeBSM and BSA (2:1 ratio).

aeBSM shows the smallest signal features and intensity with a weak broad negative peak at around 200 nm, which has been classically assigned as “random coil”. BSM(I-S), the second most pure BSM, also displays a broad negative peak in the ca. 195 – 220 region with slightly enhanced intensity, compared to aeBSM. BSM(I) showed even stronger negative peaks in the wavelength region from ca. 200 - 240 nm. The magnified view (Figure 6(b)) shows that the broad peaks have, in fact, two local minima at 210 nm and 225 nm, and are similar to the typical features of alpha helices. As a reference, far UV CD spectrum of BSA is included in the Figure 6. The CD spectra of BSM(I) and BSA resemble each other, except for the weaker intensity for BSM(I). It is also notable that the far UV CD spectra of BSM(I) can be nearly reproduced by mixing aeBSM:BSA at the ratio of 2:1, as shown in Figure 6(b). However, this does not mean that the composition of BSM(I) is simply 2:1 of aeBSM and BSA, as BSM(I) was identified to contain various low
molecular weight proteins, as determined by SDS-PAGE (Figure 5). Nevertheless, the dominant non-mucinous component to determine the far UV CD spectrum of BSM(I) appears to be BSA, judging from the similarity between the CD spectra of BSM(I) and the mixture of aeBSM:BSA. In turn, as the protein resistance of BSM(I) is distinctively different from those of BSM(I-S) and aeBSM, and it is reasonable to propose that a synergetic effect between BSM and BSA has contributed to the excellent protein resistance properties of BSM(I).

3.4. BSM-BSA composites

Inspired from the similarity of the far UV CD spectra of BSM(I) and the mixture of aeBSM+BSA at the ratio of 2:1, protein resistance of the composite molecule was tested both by OWLS and Fluorescence microscopy. As BSA was identified as an important component to strengthen the protein resistance properties of BSM, those of BSA layer alone was also assessed. The results from OWLS and Fluorescence microscopy are compared to BSM(I) in Figure 7.

![Figure 7](image_url)

Figure 7. (a) Adsorbed masses of serum onto the BSM(I), composite (aeBSM:BSA = 2:1), and BSA films as characterized by OWLS and (b) the fluorescence intensity after exposure of FITC-tagged fluorescence on top of BSM(I) and the composite films.

The resistance of the composite films or BSA against serum adsorption was clearly inferior to that of BSM(I) as characterized by OWLS. However, the resistance against fibrinogen was
comparable for BSM(I) and the composite of aeBSM:BSA films, as characterized by fluorescence microscopy experiment. Overall, the composite films of aeBSM and BSA may improve the protein resistance of aeBSM alone, but do not exactly reproduce unique nonfouling properties of BSM(I).

4. Discussion

Nonfouling surface is desired in many scientific and industrial applications, ranging from biomedical, sewage, and marine industry. In biomedical applications, as this study is relevant to, the resistance against nonspecific adsorption of proteins and bacteria is most critical. Resistance to nonspecific adsorption of proteins is particularly important for blood-contacting biosensors. Furthermore, bacterial adhesion is known to initiate from the formation of “conditioning layer”, which is made of proteins. While mucins have shown effective nonfouling properties against bacteria in literature, studies for protein resistance is very rare to date. In a previous study where purified BSM does not show extremely effective protein resistance, it was suggested that this difference might be related to the size of proteins and bacteria.

Mainly three different views have been proposed on the mechanism of nonfouling properties, especially to explain the excellent protein resistance properties of surface-grafted poly(ethylene oxide)(PEO) chains. The first view is chemical characteristics of the NSF surfaces; probably the most representative hypothesis is the “four requirements” by NSF proposed by Whiteside group in that nonfouling surfaces should be electrically neutral, hydrophilic, and possess hydrogen bond acceptors but not hydrogen bond donors. The second view is structural exclusion by NSF layer to intervene the approach and adsorption of proteins. The third view is the formation of strongly bound hydration layer on NSF layers; as well known, adsorption of protein onto a synthetic surface is proceeded mainly by entropic gain from the release of water molecules from the surface. Thus, if water molecules are somehow strongly bound to surface, and thus hard to be replaced with
proteins, protein adsorption becomes a thermodynamically unfavorable process. Of course, these three views are possibly intertwined and inter-correlated to each other a certain extent.

The exact origin and mechanism of excellent protein resistance by BSM(I) film is presently elusive. It is particularly intriguing as the other types of BSM films studied in this study do not display as effective protein resistance as BSM(I). It can be attempted though to assess the unique nonfouling properties of BSM(I) in view of three different views mentioned above. Firstly, in chemistry point of view, BSM layers are hydrophilic, but negatively charged, and very likely to carry both hydrogen bonding donor as well as acceptors. This is probably the reason why the films generate from aeBSM and BSM(I-S) do not show excellent nonfouling properties. Zeta-potential measurements have shown nearly indistinguishable values for different types of BSM solutions (ranging from ca. -5 to -8 mV for 1 mg/mL at pH 7.4). Due to the negative charges, such as from sialic acids abundant in glycans, BSM can readily attract oppositely charged proteins from serum. In this sense, it is rather unexpected and surprising that BSM(I) films repeal the adsorption of serum proteins. The structural exclusion by BSM films against protein adsorption cannot account for the difference the protein-resistant behavior of BSM(I) vs BSM(I-S) or aeBSM either. All types of BSM films tend to induce increasing amount of adsorbed masses, and presumably thicker films with increasing preparation time. But, the protein resistance appeared to be independent from the layer thickness, as long as a threshold adsorbed mass is achieved. Thus, the first two views appear to fail to explain the unique protein resistance of BSM(I) film. Instead, it can be proposed that BSM(I) may be able to form very strongly bound hydration layers compared to other types of BSMs, as suggested in the third view mentioned above. While all mucin molecules generally attract a large amount of water on the surface, the detailed mode of hydration on surface can be very different. For example, a previous QCM-D study has shown that porcine gastric mucin (PGM) forms on PDMS surface form fairly viscous and fluidic like layer, whereas BSM form has shown
more elastic and solid-like layer.\textsuperscript{21} Thus, even though the amount of water trapped within mucin layer is higher for PGM film, the water layer is more strongly bound for BSM film and displays more lubricious properties. A similar hydration strength issue may be applicable to the difference in the protein resistance between different BSM films. In near future, experiments to characterize the binding strength of water around different BSM films are to be conducted.

5. Conclusions

In this study, we have demonstrated that the films generated from BSM(I) on a hydrophobic surface reveal unique protein resistance, such as serum and fibrinogen, whereas not equally effective protein resistance properties were observed from BSM(I-S) or aeBSM films. The major difference of BSM(I) compared to the others has been determined to be abundant various small proteins other than BSM. A composite of aeBSM and BSA at the ratio of 2:1 could not exactly reproduce the excellent protein resistance of BSM(I) films, suggesting that other protein components than BSA may be necessary to reconstruct BSM(I) films. As BSM(I) film is a unique polyanionic molecule, neutrality cannot be a part of driving forces that impart the nonfouling properties. Formation of stronger hydration layer is proposed as a possible mechanism for BSM(I) being superior in protein resistance, but this model has to be verified experimentally.

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