Influence of Glycosylation on Interfacial Properties of Recombinant Mucins: Adsorption, Surface Forces and Friction

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ABSTRACT

Interfacial properties of two brush-with-anchor mucins, C-P55 and C-PSLex, have been investigated at the aqueous solution/poly(methylmethacrylate) (PMMA) interface. Both are recombinant mucin-type fusion proteins, produced by fusing the glycosylated mucin part of P-selectin glycoprotein ligand-1 (PSLG-1) to the Fc part of a mouse immunoglobulin in two different cells. They are mainly expressed as dimers upon production. Analysis of the O-glycans shows that the C-PSLex mucin has the longer and more branched side chains, but C-P55 has slightly higher sialic acid content. The adsorption of the mucins to PMMA surfaces was studied by quartz crystal microbalance with dissipation. The sensed mass, including the adsorbed mucin and water trapped in the layer, was found to be similar for these two mucin layers. Atomic force microscopy with colloidal probe was employed to study surface and friction forces between mucin-coated PMMA surfaces. Purely repulsive forces of steric origin were observed between mucin layers on compression, whereas a small adhesion was detected between both mucin layers on decompression. This was attributed to chain entanglement. The friction force between C-PSLex-coated PMMA is lower than that between C-P55-coated PMMA at low loads, but vice versa at high loads. We discuss our results in terms of the differences in the glycosylation composition of these two mucins.
1. INTRODUCTION

Mucins are the major component of mucus, exist in secreted and membrane-bound forms, and coat almost all wet surfaces in the mammalian body. Mucins, together with other components in mucus, provide anti-adhesion, protection, hydration and lubrication functions.\(^1\) Although the composition and structures of mucins vary depending on their origin, they display certain common features.\(^2\), \(^3\), \(^4\) Many mucins are composed of heavily glycosylated domains separated by short non-glycosylated or sparsely glycosylated patches, forming train-of-brushes structures. The glycosylated domains contain heterogeneous oligosaccharides chains, which are usually short and often branched.\(^5\) For example, the most commonly studied bovine submaxillary mucin (BSM) contains 19 different types of acidic or neutral \(O\)-linked carbohydrate side chains.\(^6\) The oligosaccharide chains are usually built from sugar units such as, GlcNAc, GalNAc, Gal, Fuc and sialic acids.\(^1\), \(^2\) The size of the oligosaccharide chains of different mucins varies from 2 - 5 sugar units per chain to 16 - 19 sugar units per chain depending on the source.\(^7\)

The ability of mucins to adsorb onto different surfaces is of great importance in living tissues and biomaterial applications.\(^8\) Hence, there have been extensive studies regarding the performance of mucins in contact with surfaces.\(^9\), \(^10\), \(^11\), \(^12\), \(^13\) Mucins can adsorb to various surfaces, rendering the underlying substrate hydrophilic and lubricative.\(^4\) When adsorbing to hydrophobic surfaces from an aqueous solution, it is the non-glycosylated, hydrophobic domains that bind to the surface via hydrophobic interactions, while the glycosylated, hydrophilic domains stretch out into aqueous media due to favorable interactions with water.\(^9\) Thus, the majority of train-of-brushes mucins adsorbed on hydrophobic surfaces have an orientation preferentially parallel to the surface, with the side chains forming a brush structure. However, this does not exclude some tails extending further from the surface.\(^10\), \(^11\), \(^12\) For example, the thickness of the adsorbed BSM layer on hydrophobic surfaces is in the range of
4-7 nm\textsuperscript{13,14,15,16}, which is close to the thickness of the glycosylated backbone chain (estimated to be 6 nm\textsuperscript{13}). When surfaces bearing such mucin layers slide across each other, the brush structure of large regions of these molecules will counteract chain interpenetration, which is favorable for achieving good lubrication properties. Furthermore, the steric and electrostatic forces generated by the glycosylated domains of mucins and the high hydration level of these domains are of importance for the lubricity of mucin-coated surfaces.\textsuperscript{3} For instance, Yakubov et al. reported that the adsorption of ‘Orthana mucin’ on hydrophobic poly(dimethylsiloxane) (PDMS) surfaces led to a 10-fold reduction in boundary friction.\textsuperscript{17} Another study utilizing ‘Orthana mucin’-coated hydrophobized mica surfaces was reported by Harvey et al. They achieved a coefficient of friction ($\mu_{\text{cof}}$) as low as 0.01-0.02 (up to a pressure of 0.7 MPa). The favorable lubrication property of this mucin layer was ascribed to the absence of bridging and large number of hydrophilic moieties exposed to the solution as a result of the brush structure of the adsorbed mucin.\textsuperscript{18} Our previous study on the lubrication ability of two mucins with different structures also shows that the mucin-type immunoglobulin fusion protein, PSGL-1/mIgG2b, with a brush-with-anchor structure\textsuperscript{1} lubricates PMMA significantly better ($\mu_{\text{cof}} \approx 0.02-0.24$) than BSM with a train-of-brushes structure ($\mu_{\text{cof}} \approx 0.7$). The lower $\mu_{\text{cof}}$ of PSGL-1/mIgG2b was explained by less importance of bridging forces and strong binding between the anchor group and PMMA.\textsuperscript{14}

The glycosylated domains of mucins are suggested to strongly affect the interfacial properties; however, it is difficult to discuss the structure-property relations in detail.\textsuperscript{4,18} The reason is that most commercial mucins contain other proteins and the presence of impurity affects its interfacial properties.\textsuperscript{19,20} Herein we are able to fill this gap by utilizing the highly purified recombinant mucins, C-P55\textsuperscript{21} and C-PSLex\textsuperscript{22}, with controlled structural differences

\textsuperscript{1} The brush-with-anchor mucin has a distinct anchor group with no or low glycosylation from which two glycosylated chains extend, see Figure 1.
and similarities, including the same peptide backbone and similar anchoring group, but different oligosaccharide side chains. The purpose of this investigation is to elucidate how the glycosylation composition, including sialic acid content and oligosaccharide chain length, affects the interfacial properties of these two recombinant mucins. The interfacial properties of C-PSLex and C-P55 on PMMA, including the adsorption behavior, surface and friction forces have been investigated using quartz crystal microbalance with dissipation (QCM-D) and atomic force microscopy (AFM) with colloidal probe. The different interfacial properties of these mucins are discussed and related to their structural differences.

2. MATERIALS AND METHODS

2.1. Materials

The recombinant mucin-type fusion protein PSGL-1/mIgG2b created by fusing the cDNA encoding the extracellular part of P-selectin glycoprotein ligand-1 (PSGL-1) to the Fc part of mouse IgG2b was produced in Chinese hamster ovary (CHO) cells as described previously. The secreted PSGL-1/mIgG2b contains 538 amino acids with a theoretical molecular weight of 58 kDa. PSGL-1/mIgG2b produced in the cell clones C-PSLex and C-P55 are in this article named C-PSLex and C-P55 mucins, respectively. We note that the C-PSLex mucin was referred to as PSGL-1/mIgG2b in our previously publication. C-PSLex and C-P55 are mainly produced as dimers, and the schematic mucin structures are shown in Figure 1. C-PSLex and C-P55 have the same peptide sequence and each dimeric fusion protein has 106 potential O-glycan sites and 6 potential N-glycan sites on the mucin part. However, even if the protein backbone is the same in these two mucins, the carbohydrate structures attached to the backbone vary depending on the host cells, C-PSLex or C-P55, as detailed in the results section. The apparent molecular weight of these two mucins is around 300 kDa as estimated by SDS-PAGE chromatography and depends on the degree of glycosylation and sialylation/sulfation. The sialic acid content, NeuAc and NeuGe, in the two mucins was determined by the
Glycotechnology Core Resource, University of California, San Diego. The detailed description of the analyzing method is provided in the Supplementary Information. Mucin stock solutions of 200 ppm were prepared by dissolving weighed portions of mucin in NaCl solution. All solutions were stored at 4 °C for at most 3 days and diluted with 155 mM NaCl solution prior to use. The pH of the NaCl, C-PSL Lex and C-P55 solutions was 5.8, 4.8 and 4.7, respectively. The pKa of sialic acid is around 2.6, and under the experimental condition both mucins are negatively charged. Sodium chloride (NaCl, BioXtra, ≥ 99.5%) was purchased from Sigma-Aldrich and used as received. All water used was purified to a resistivity of 18.2 MΩ cm by employing a Milli-Q Purification System (Millipore, Malsheim, France) and the total organic carbon content of the water did not exceed 2 ppb.

![Figure 1](image.png)

**Figure 1.** Schematic illustration of the predominant O-glycan structures of the recombinant mucins PSGL-mIgG2b produced in the CHO cell clone C-P55 and C-PSL Lex. The figure is not drawn to scale. The molecular structures of the carbohydrate units listed in the figure are shown in the Supplementary Information, Figure S1.

Poly(methylmethacrylate), PMMA, coated gold sensors QSX 999 (AT cut quartz crystals Q-sense, Västra Frölunda, Sweden), were used as the substrate for both QCM-D and AFM experiments. The water contact angle on the PMMA surface was around 68°. More detailed description about the substrate and the cleaning method can be found elsewhere.14

### 2.2. Liquid Chromatograph-tandem Mass Spectrometry (LC-MS/MS)

**Characterization of Released N- and O-glycans**
C-P55 and C-PSLex (300 μg) were solubilized in 50 mM sodium phosphate buffer, pH7.5. The samples were reduced with 25 mM DTT at 95°C for 10 min. The N-glycans were released by incubation with N-glycanase F (PNGase F, 5 units, ProZyme, Hayward, CA) at 37°C overnight. Non-reductive N-glycans were separated from the remaining glycoproteins using Pall Nanosep® spin-filters with 10-kDa cutoff (Pall, Port Washington, NY). The released N-glycans were reduced by incubation with 0.5 M NaBH₄ and 20 mM NaOH at 50°C overnight. The O-glycans were subsequently released from the glycoproteins by adding an equal volume of 1.0 M NaBH₄ and 100 mM NaOH on top of the filters and incubating at 50°C overnight. The released O-glycans were separated from remaining by centrifugation through the Pall Nanosep® spin filter. Both N- and O-glycans were desalted and dried as previously described.

Reduced N- and O-glycans were analyzed by LC-MS/MS using a 10 cm × 250 μm inner diameter column, prepared in-house, containing 5 μm porous graphitized carbon (PGC) particles and an LTQ mass spectrometer (Thermo Scientific, Waltham, MA) in negative-ion mode with an electrospray voltage of 3.5 kV, capillary voltage of -33.0 V, and capillary temperature of 300°C. Compressed air was used as sheath gas. In LC-MS/MS, full-scans were performed in the mass range of m/z 380-2000, and at normalized collisional energy of 35% with a minimal signal of 300 counts, isolation width of 2.0 m/z, and activation time of 30 ms. Glycans were eluted using a linear gradient from 0-40% acetonitrile in 10 mM ammonium bicarbonate over 40 min at a flow rate of 10 μL/min. The data were processed using the Xcalibur software (version 2.0.7, Thermo Scientific).

2.3. Quartz Crystal Microbalance with Dissipation (QCM-D)

A Q-sense E4 device (Q-sense, Sweden) was employed for studying adsorption of mucins on PMMA surfaces. All QCM-D experiments were started by obtaining a stable baseline in 155 mM NaCl solution. Next, 100 ppm mucin solution was pumped into the QCM-D chamber
with a flow speed of 50 µL/min by using a peristaltic pump. After the adsorption reached equilibrium, the mucin solution was rinsed by pumping 155 mM NaCl solution into the chamber until stable frequency and dissipation signals were obtained. All experiments were performed at 25 ± 0.2 °C. The changes in frequency (Δf) and dissipation (ΔD) at the fundamental frequency as well as at six different overtone frequencies (15, 25, 35, 45, 55, 65 MHz) were recorded. The frequency change observed during adsorption depends on the total mass added to the sensor, including solvent coupled to the adsorbed layer. The dissipation change is due to energy losses in the adsorbed film when it oscillates together with the sensor.

In our experiments the dissipation changes due to mucin films formation are significant and the change in frequency normalized by overtone number for the different overtones does not overlap. This demonstrates that the viscoelastic properties of the adsorbed layer affect the sensor response, and therefore the Sauerbrey equation\(^26\) is not valid for calculating the adsorbed mass. Instead, we applied the Voigt model where changes in both frequency and dissipation for several overtones are utilized when analyzing the data.\(^{26, 27}\) Furthermore, when, as in our case, the viscoelastic properties of the adsorbed film are frequency-dependent an extended viscoelastic model that considers this should be used. A detailed description of the extended viscoelastic model and its applicability have been published elsewhere.\(^{27}\) In this study, the experimental data were analyzed using the extended viscoelastic Voigt model by utilizing frequency and dissipation data from the 3rd, 5th, 7th and 9th overtones. The modeling parameters used for analyzing the adsorption of C-PSLex on PMMA has been published elsewhere\(^{14}\) and the same parameters were also used in the analysis of adsorbed C-P55 layers. The parameters are also provided in the Supplementary Information, Table S2. The modelling quality obtained by utilizing the viscoelastic and, alternatively, the extended viscoelastic model is shown in Figure S2.

### 2.4. Atomic Force Microscope (AFM) with Colloidal Probe
Force and friction measurements were determined by utilizing a Nanoscope Multimode 8 Pico Force AFM (Bruker, USA). The *in situ* experiments were performed in a fused silica liquid cell (volume $\approx 0.1$ mL) using rectangular tipless cantilevers (MikroMasch, CSC12/tipless/Cr-Au) with the approximate dimensions of 250 μm in length, 35 μm in width, and normal spring constants in the range 0.02 – 0.2 N/m. A spherical PMMA particle (Kisker, cat.#ppmma-10.0) with a diameter of approximately 10 μm was attached to the end of the cantilever with a small amount of epoxy glue (Araldite, 80806) to be used as a colloid probe. The attachment was done with the aid of an Ependorf Micromanipulator 5171 and a Nikon Optiphot 100S reflection microscope. The exact values of the normal ($k_N$) and the torsional ($k_\phi$) spring constants of the cantilevers were determined before the colloidal probe was attached by using the AFM Tune IT v2.5 software (Force IT, Sweden) adopting the method based on thermal noise with hydrodynamic damping.\textsuperscript{28, 29} Before each experiment, the fused silica cell and all other tools were cleaned by immersion in 2% Hellmanex (Hellma GmbH) solution for 1 hour and then rinsed excessively with Milli-Q water. They were then rinsed with ethanol before being dried with a stream of filtered nitrogen gas. The cantilevers, each carrying an attached colloidal probe, were rinsed with water and ethanol prior to use. The lateral photodetector sensitivity ($\delta$, V/rad) was calibrated using the method of tilting the AFM head proposed by Pettersson et al.,\textsuperscript{30} and a measured value of 4670 V/rad was used in the calculation of the friction forces.

The AFM experiments were started by measuring the normal forces acting between the PMMA surface and the PMMA colloidal probe across a 155 mM NaCl solution, and followed by friction measurements. The force curves were measured with a constant approach and retraction speed of 1 μm/s. In each case at least 10 curves were recorded. The force curves were analyzed with the AFM Force IT software (Force IT). Friction forces were evaluated by scanning the colloidal probe perpendicular to the long axis of the cantilever and measuring the
lateral deflection of the cantilever. The friction forces were measured at different applied loads (from the onset of the steric repulsion up to the highest load applied and again on decreasing the load) at a sliding speed of 4 μm/s with a scan size of 2 μm. At least 10 measurements were performed at each load. The friction traces obtained were analyzed by employing the AFM Friction IT software (Friction IT). The force and friction measurements between mucin layers were performed after allowing the adsorption from a 100 ppm mucin solution to proceed for around 45 minutes. The deflection sensitivity obtained for the bare PMMA-PMMA system was used when defining the constant compliance region and generating force curves in the presence of adsorbed mucin layers. The force and friction measurements were also done after rinsing with 155 mM NaCl, approximately two hours after injecting the mucin solution. All measurements were carried out at 25 °C.

3. RESULTS

3.1. Carbohydrate Composition of C-PSLex and C-P55

The recombinant C-PSLex and C-P55 mucins share some similarities. Both have the same protein backbone, which is from the extracellular part of PSGL-1 (291 amino acids), and similar anchoring group, the Fc part of mouse IgG2b (239 amino acids). The Fc part, as a dimer, contains two N-glycans; while the PSGL-1 part is heavily O-glycosylated. Monomers of both mucins migrate similarly on SDS-PAGE (10%) with apparent molecular weight of 130 kDa (Figure 2a). After removal of N-glycans by PNGase F, the size of both mucins was only reduced slightly. However, desialylation of both mucins results in a dramatic decrease in their mass to around 50 kDa according to the prestained protein marker (Figure 2a). This demonstrates that both mucins are heavily sialylated, and that most sialylation occurs on the O-glycans reflecting their mucin-like properties. Thus, the two recombinant mucins differ from each other with respect to the degree of sialylation and the composition of the oligosaccharide chains.
The sialic acid content of these two mucins is shown in Table 1. The sialic acid content of C-P55 is slightly higher than that of C-PSLex, but considering the high ionic strength of our measurements (155 mM) we do not expect differences in electrostatic interactions to play a major role. Mass spectrometric analysis reveals that the relative amount of sialylated O-glycans from both samples is similar. However, the relative amount of sialylated N-glycans (including 6 potential N-glycan sites on the mucin part and the 2 N-glycans on the Fc part) from C-P55 (36% of total N-glycans on C-P55) is higher than that of C-PSLex (19% of total N-glycans on C-PSLex, Supplementary Information Table S3.)

In addition, the size, as determined by glycan chain length and mass, of the two mucins is also different (Figure 2b). Both weighted average glycan chain length and mass of O- and N-glycans are calculated using the relative abundance of each glycan (Supplementary Information Table S3 and S4). Both C-P55 and C-PSLex have similar N-glycan chain length (9.1 and 9.4 residues, respectively) and mass (1681.6 and 1714.8 Da, respectively). However, C-PSLex is decorated with on average longer O-glycan chains (5.2 versus 3.9 residues) with larger mass (1038.4 versus 741.3 Da).

The stable CHO cell clone C-P55 utilized for production of one of the mucins is not glycoengineered and thus generates a mucin carrying O-glycans that reflects the natural glycan

### Table 1. Sialic Acid Content for C-P55 and C-PSLex Mucins

<table>
<thead>
<tr>
<th>Sample</th>
<th>Neu5Gc</th>
<th>Neu5Ac</th>
<th>Total</th>
<th>Wt % (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-P55</td>
<td>0.5</td>
<td>157.1</td>
<td>157.6</td>
<td>15.8%</td>
</tr>
<tr>
<td>C-PSLex</td>
<td>0.6</td>
<td>140.3</td>
<td>140.9</td>
<td>14.1%</td>
</tr>
</tbody>
</table>

Note: The analysis of the C-PSlex and C-P55 also suggested the presence of a small amount of acetylated Neu5Ac, namely Neu5,8Ac2 and Neu5,9Ac2.
phenotype of CHO cells. As a result, C-P55 carries mainly mono- and disialylated core 1\textsuperscript{ii} O-glycans. Mass spectrometric analysis of chemically released O-glycans from C-P55 reveals a quite simple glycan phenotype with major peaks (Figure 2d) at \(m/z\) 675.58 (53.5%) and \(m/z\) 966.50 (17.9%), representing monosialylated (NeuAcα2,3Galβ1,3GalNAcβ1-) and disialylated (NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAcβ1-) core 1, respectively. In contrast, the CHO cell clone C-PSLex is co-transfected to stably express \(\beta1,6\)-N-acetylglucosaminyltransferase I (core 2\textsuperscript{iii} GnT I) and α1,3-fucosyltransferase VII (FucT VII). The glycan phenotype of O-glycans released from C-PSLex is consequently more complex. The main mass spectrometric peaks are noted at \(m/z\) 1040.67 (32.4%) and \(m/z\) 1331.58 (9.2%) representing monosialylated core 2 with NeuAc on the C3 branch (Galβ1,4GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcβ1-) and disialylated core 2 with NeuAc on the C3 and C6 branches (NeuAcα2,3Galβ1,4GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcβ1-), respectively (Figure 2d).

As for the N-glycans, mass spectrometric analysis of enzymatically released N-glycans from C-P55 revealed that the dominant N-glycans were core fucosylated bi-antennary with one (19.7%) or two (11.8%) terminal Gal residues or one NeuAc residue (11.7%) with \(m/z\) values at 1625.62 ([M−H]⁻) or 812.60 ([M−2H]²⁻) 1787.67 ([M−H]⁻) or 893.55 ([M−2H]²⁻), and 1039.59 ([M−2H]²⁻), respectively (Figure 2e). In contrast, the dominant N-glycans from C-PSLex are mostly neutral with no (16.3%), one (23.1%) or two (25.3%) terminal Gal residues with \(m/z\) values at 1463.64 ([M−H]⁻) and 731.54 ([M−2H]²⁻), 1625.64 ([M−H]⁻) and 812.61 ([M−2H]²⁻), and 1788.67 ([M−H]⁻) and 894.18 ([M−2H]²⁻), respectively (Figure 2e). Detailed putative N- and O-glycans structures and their relative abundance are listed in Supplementary Information Table S3 and S4.

\textsuperscript{ii} O-linked oligosaccharides with the structure of Galβ1,3GalNAcβ1-O-Ser/Thr.

\textsuperscript{iii} O-linked oligosaccharides with the structure of GlcNAcβ1,6(Galβ1,3)GalNAcβ1-O-Ser/Thr.
Figure 2. Characterization of the glycan profile of C-P55 and C-PSLex recombinant mucins. (a) SDS-PAGE (8%) analyzing reduced (M), N-glycan depleted (dN), or desialylated (dSia) mucins. Recombinant mucins are indicated by arrowhead. (b and c) Average number of residues and mass of glycans released from recombinant mucins. Values corresponding to the weighted average glycan residues and masses of glycan structures on each mucin were calculated using the relative abundance of each glycan. (d and f) LC-MS/MS analysis of released O- and N-glycans from the recombinant mucins. The symbols are the same as in
Figure 1. Proposed structures are depicted using the Symbol Nomenclature for Glycomics (SNFG). Glycans were manually annotated from their MS/MS spectra and validated by available structures stored in the UniCarb-DB database (2016.09 version).

To summarize, the glycan phenotype of recombinant mucin C-PSLex is represented by longer and more branched O-glycan structures than that found of the C-P55 mucin (Figure 2), and the relatively few N-glycans have a higher degree of sialylation on C-P55 than on C-PSLex. Although, the glycophenotype of the fusion proteins are heterogeneous, the glycan phenotypes of recombinant mucins produced in C-P55 and C-PSLex also have similarities; both are heavily sialylated and thus negatively charged.

### 3.2. Adsorption of Mucins on PMMA

The adsorption of the C-P55 mucin onto a PMMA surface monitored by QCM-D is shown in Figure 3a. The QCM-D response is rather complex. Initially there is a rapid decrease in frequency and increase in dissipation suggesting a rapid first step in the formation of a viscoelastic mucin layer. The following evolution of the layer is much slower; a minimum in frequency of 65 Hz is reached just before 1.5 hours (when the flow was stopped) while the dissipation increases slightly. This is followed by a slow frequency increase, about 5 Hz, while the dissipation is largely unaffected. During rinsing, the frequency increases further and stabilizes at around 50 Hz and the dissipation decreases by about $0.5 \times 10^{-6}$. The rather complex evolution of $\Delta f$ and $\Delta D$ shown in Figure 3a is reproducible between experiments, as can be seen from the $\Delta D-\Delta f$ plots in Figure 3b, and one can distinguish four regions in the $\Delta D-\Delta f$ plot, corresponding to the regions with the same number in Figure 3a. We attribute the complex evolution with time to two processes that occur under similar time scales: conformational rearrangement in the adsorbed layer to minimize the free energy and additional adsorption of some mucin molecules into the layer. It is interesting to see that there is a plateau region between 53 and 58 Hz, region 2, in which the dissipation stops increasing or even decreases.
slightly while the frequency continuously decreases. This phenomenon has to the best of our knowledge not been reported previously. We suggest that it is due to conformational and translational relaxation of mucin molecules after their arrival to the surface, optimizing chain conformations and relative positions on the surface. Afterwards, the dissipation increases as frequency decreases (region 3), but with a slightly lower $\Delta D/\Delta f$ ratio compared to region 1. We attribute this to the mucin layer becoming slightly more extended as a few additional molecules attach to the surface. In region 4 (the region shown more clearly in the inset), the dissipation decreases with increasing frequency, which was also observed in the $\Delta D$–$\Delta f$ plot for BSM, resulting from the rearrangement of the C-P55 molecules and possibly leading to removal of less strongly adsorbed molecules.

The comparison of the $\Delta D$-$\Delta f$ curves obtained on adsorption of C-P55 and C-PSLex on PMMA is shown in Figure 3c. The two curves differ significantly and regions 2 and 4 exist only for the C-P55 mucin. The different behavior of these two mucins upon adsorption should be attributed to the different O-glycan repertoires on the two mucins, with shorter side chains on C-P55.
Figure 3. (a) Frequency (filled symbol) and dissipation (open symbol) changes from the 5\textsuperscript{th} overtone with time during adsorption from 100 ppm C-P55 mucin solution in 155 mM NaCl on PMMA. The arrow labeled with C-P55 marks the starting point of mucin solution injection. (b) Three $\Delta D\text{-}\Delta f$ plots of C-P55 adsorption on PMMA. The inset shows an enlargement of region 4 of the red curve. (c) Comparison of $\Delta D\text{-}\Delta f$ plots for the two mucins, C-P55 (open circles) and C-PSLex\textsuperscript{14} (filled circles).

Some C-P55 and C-PSLex mucin layer characteristics obtained from the QCM-D data are summarized in Table 2. We particularly note that the sensed mass decreases for C-P55 upon rinsing due to some desorption, whereas it increases for C-PSLex due to swelling.

Table 2. Characteristics of C-P55 and C-PSLex layers on PMMA evaluated from the QCM-D adsorption experiments shown in Figure 3.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta f/5$ (Hz)</th>
<th>$\Delta D$ ($10^{-6}$)</th>
<th>Voigt thickness (nm)</th>
<th>Voigt mass (mg/m\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thickness</td>
<td>Voigt Mass</td>
<td>Voigt Mass (mg/m²)</td>
<td>Voigt Mass (mg/m²)</td>
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<tr>
<td>--------</td>
<td>-----------</td>
<td>------------</td>
<td>--------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>C-P55</td>
<td>62.1 ± 1.0</td>
<td>6.1 ± 0.2</td>
<td>18.1 ± 0.5</td>
<td>19.4 ± 0.7</td>
</tr>
<tr>
<td>after rinsing</td>
<td>50.3 ± 0.8</td>
<td>5.6 ± 0.2</td>
<td>16.5 ± 0.5</td>
<td>17.8 ± 0.6</td>
</tr>
<tr>
<td>C-PSLex</td>
<td>59.8 ± 0.2</td>
<td>6.6 ± 0.2</td>
<td>18.9 ± 0.2</td>
<td>20.4 ± 0.2</td>
</tr>
<tr>
<td>after rinsing</td>
<td>58.9 ± 0.5</td>
<td>7.4 ± 0.2</td>
<td>20.4 ± 0.2</td>
<td>22.0 ± 0.2</td>
</tr>
</tbody>
</table>

The standard deviation was calculated based on three experiments. The Voigt thickness and Voigt mass were obtained from the extended Voigt model using data from the 3rd, 5th, 7th and 9th overtones. The data marked with * were reported in our previous publication. Note that the Voigt mass includes both the mass of the adsorbed mucin and the mass of water associated with the layer.

The thickness extracted from the QCM-D results for both C-P55 and C-PSLex layers is significantly smaller than the length of the mucin domain, which is around 50 nm. Thus, the adsorbed mucin domains have, on average, an orientation that is significantly tilted relative to the surface normal. We chose to describe the conformation as tilted rather than coiled due to the stiff nature of glycosylated mucin domains.

### 3.3. Forces between Mucin-coated PMMA Surfaces

The forces acting between C-P55-coated PMMA as a function of separation are shown in Figures 4a and b. On approach a purely repulsive steric force is encountered between C-P55 layers (electrostatic double-layer forces are suppressed due to the high ionic strength, 155 mM).

The steric origin of the long-rang force measured between mucin coated surfaces has also been reported previously. However, a small but clearly visible attraction is observed on separation even though the sensed mass is not low. The hysteresis between forces measured on approach and separation suggests that the relaxation processes in the layer are not fully reversible during the time scale of the measurement (the layers stay in contact for about 0.8 s during measurement of one approach and retraction force curve). Comparison of the forces acting between C-P55-coated PMMA before and after rinsing show no obvious variation despite that the QCM-D results indicate that a small amount of C-P55 was removed upon rinsing (the Voigt mass decreases from 19.4 to 17.8 mg/m², see Table 1). This suggests that the segment density in the dilute tail region was not significantly changed upon rinsing, since...
the repulsion at large separation mainly arises from the osmotic pressure arising from the segment density difference at the midpoint between the surfaces and the bulk solution at high ionic strength.\textsuperscript{36}

In contrast, the forces measured between C-PSLex-coated PMMA differ before and after rinsing as shown in Figures 4c, d, and f, respectively. The steric force encountered on approach is less long ranged after rinsing. Further, the attractive force experienced on decompression starts from a shorter separation, around 75 nm after rinsing compared to about 110 nm before rinsing. These results may at first seem to be at odds with the QCM-D data that suggests that the C-PSLex layer swells upon rinsing. However, one needs to bear in mind that the AFM colloidal probe technique, unlike the surface force apparatus, does not provide a measure of the thickness of the layer. Thus, the thickness of the layer at the defined zero distance in Figure 4 is not known, and the force data should be interpreted as showing that the tails extend less from the overall swollen mucin layer after rinsing.
Figure 4. (a) Ten force-separation curves measured on approach between C-P55-coated PMMA before (black) and after rinsing (red). (b) Ten force-separation curves measured on separation between C-P55-coated PMMA before (black) and after rinsing (red). (c) Ten force-separation approach curves between C-PSLex-coated PMMA before (black) and after rinsing (red). (d) 10 force-separation curves determined on separation between C-PSLex-coated PMMA before rinsing. (e) Force-separation curves between C-PSLex-coated\textsuperscript{14} (open circles) and C-P55-coated PMMA (filled circles) measured on approach before rinsing. (f) Ten force-separation curves measured on separation between C-PSLex-coated PMMA after rinsing. The mucin
concentration was 100 ppm prior to rinsing and the NaCl concentration was 155 mM in all cases. All forces are normalized by the particle radius.

The forces measured between C-PSLex layers and C-P55 layers on approach are compared using a logarithmic force scale in Figure 4e. These two force curves resemble each other, even though the repulsion is stronger for C-PSLex. Both of them increase exponentially with decreasing separation in the range 50 nm to 5 nm with a decay length of around 22 nm. This suggests similar segment density distribution in the dilute tail region. At small separation, < 5 nm, the steric forces increase more rapidly due to further compression of the adsorbed mucin layers that reduces the conformational entropy. The attraction observed on separation is more long-ranged for C-PSLex-coated PMMA than for C-P55-coated PMMA (compare Figures 4b, d and e). It appears that the longer side chains of C-PSLex facilitate entanglement and the layers need to be separated further before they completely detach.

3.4. Friction Forces

The friction force between C-P55-coated PMMA before and after rinsing increases roughly linearly with load after the layers have come in close contact, i.e. when F/R is about 2 mN/m and above (Figure 5a). Thus, Amontons’ first rule that states that the friction force is proportional to the load, describes this system well except at very low loads. This also means that the effective friction coefficient, \( \mu_{\text{eff}} \) defined as: \( \mu_{\text{eff}} = \frac{F_f}{F_n} \) is close to constant (Figure 5b). In addition, the friction forces measured before and after rinsing are similar for C-P55-coated PMMA.
Figure 5. a. Friction force $F_f$ vs. load, $F_n$ and $F_n/R$, between C-PSLex (red squares: before rinsing\textsuperscript{14}; blue squares: after rinsing.) and C-P55 (black circles: before rinsing; black squares: after rinsing) coated PMMA. Filled and unfilled symbols represent data obtained on loading and unloading, respectively. b. The effective friction coefficient $\mu_{\text{eff}}$ vs. load (average between loading and unloading curves) for C-PSLex (red square: before rinsing\textsuperscript{14}; blue square: after rinsing) and C-P55 (filled circles: before rinsing; open circles: after rinsing) coated PMMA. The mucin concentration was 100 ppm and the NaCl concentration was 155 mM in all cases. The error bars are the standard deviation based on 10 measurements at each load.

In contrast, the friction force between C-PSLex mucin layers before rinsing is lower than that observed between C-P55 mucin layers at loads $< 4$ mN/m, which corresponds to a pressure of around 6 MPa, calculated by Hertz theory\textsuperscript{37} using a Young’s modulus of 5 GPa\textsuperscript{38} and a Poisson’s ratio of 0.38\textsuperscript{39} for PMMA. However, at higher loads the friction force between C-PSLex-coated PMMA is higher than observed between C-P55-coated PMMA. Thus, for surfaces coated with C-PSLex mucin Amontons’ first rule is not applicable as illustrated in Figure 5b. The existence of a low-friction region in the friction vs load curve has also been reported for another mucin\textsuperscript{18}, lubricin\textsuperscript{40} and for some synthetic bottle-brush polymers\textsuperscript{41}. After rinsing, the friction force between C-PSLex-coated PMMA in the low friction region is similar to that between C-P55-coated PMMA when $F_n/R < 4$ mN/m; but higher when $F_n/R$ is above
this value. In addition, we note that the friction force between C-PSLex-coated PMMA before rinsing is higher than that after rinsing when $F_a/R > 4 \text{ mN/m}$.

The effective friction coefficient between C-P55-coated PMMA is around 0.1 when the applied normalized force is above 1 mN/m, which is higher than the friction coefficient of the low-friction region of C-PSLex-coated PMMA but lower than that of the region after the transition point, with a limiting value of 0.24. Thus, at the highest pressure applied, which is about 8 MPa, the C-P55 layers provide superior lubrication of PMMA surfaces in aqueous environment compared to C-PSLex, but inferior performance at loads < 6 MPa. However, both mucins are able to provide excellent lubricity under conditions experienced by contact lenses, in which the highest applied pressure is 1-3 kPa$^{42}$, corresponding to a force of only $1 \times 10^{-9} \text{ nN}$ in our experimental set-up. The friction coefficients determined for porcin gastric mucin coated hydrophobized mica (0.15 to 0.3, at pressures above 0.8 MPa)$^{18}$ and lubricin coated hydrophobic alkanethiol surfaces (0.267, at loads above 0.4 mN corresponding to a pressure of about 0.6 MPa)$^{40}$, are larger than we observe at corresponding pressures (0.6-0.8 MPa corresponds to a load of 0.02-0.05 nN in our experiment).

4. DISCUSSION

4.1. Differences between C-P55 and C-PSLex Recombinant Mucins

The two recombinant mucins investigated in this study are both of the brush-with-anchor type, where the adsorbing Fc part is separated in space from the glycosylated mucin part. Since the anchoring group is similar for these two mucins, the differences observed are predominantly related to their different glycosylation in the mucin domains. Here the distinguishing features are that i) the C-PSLex mucin has longer O-glycan side chains (typically 5 or 6 carbohydrate units, compared to 3 or 4 units for C-P55, see Figure 2), and ii) the O-glycans on C-PSLex are more heterogenous (the carbohydrate unites vary from 3 to 8) and around 28% of the O-glycans consist of more than 6 carbohydrate units. As for the most
studied commercially available BSM, which was also used in our previous study,\textsuperscript{14} 49% of the oligosaccharide chains have only 2 sugar units and only 7% of them have more than 4 sugar units. The oligosaccharide chains of BSM used in our previous work are also available in the Supporting information Table S5.

4.2. The Effect of Carbohydrate Composition on Adsorption

In a previous work Pasche et al. studied the effect of side chain length on adsorption properties of polylysine-graft-poly(ethylene oxide), PLL-g-PEO, on Nb$_2$O$_5$ surfaces, and concluded that the adsorption is governed by the balance between electrostatic attraction anchoring the positively charged PLL backbone to the surface and steric repulsion arising from PEG-PEG side chain interactions.\textsuperscript{43} In a theoretical modeling study, the effect of side-chain length on the adsorption of bottle-brush polymers was investigated by Linse and Claesson.\textsuperscript{44} The results showed that a shorter side chain resulted in a more compact adsorbed polymer layer even when the surface excess is similar. Based on these findings one would expect that C-P55, having shorter side chains than C-PSLex, would adsorb in a more compact conformation than C-PSLex. The QCM-D results indicate that this is the case, where a slightly thicker layer with higher viscoelastic properties (larger $\Delta D$) is formed by C-PSLex. However, the difference is small, suggesting that the adsorption via the anchor group is not strongly affected by the O-glycan side-chain length on the mucin parts, even though the formation of the layer follows different routes for the two recombinant mucins (Figure 3c).

4.3. The Effect of Carbohydrate Composition on Surface Forces

The forces measured on approach between C-P55-coated PMMA and C-PSLex-coated PMMA are in both cases dominated by a steric force that increases exponentially with decreasing separation. The repulsion is slightly larger for the recombinant mucin with longer side-chains (C-PSLex) even though the decay length of the force is similar in both cases (see Figure 4e). On separation the attractive force extends to larger distances for C-PSLex than that
for C-P55 (compare Figures 4b, d and e). Long-range attractive forces can be caused by bridging, where a polymer chain attaches to two surfaces as has been suggested for other mucin-coated surfaces. However, for our brush-with-anchor type of mucin, bridging formed by molecules adsorbing to opposing surfaces is less likely due to the high adsorbed mass. We rather interpret the attraction as being caused by entanglement, where upon compression the mucin layers partly penetrate into each other; and the disentanglement rate upon separation is slow compared to the time scale of the measurement (the surfaces stay in contact for about 0.8 s). The longer side chains of C-PSLex would make disentanglement more difficult, and we propose that this is the reason that C-PSLex-coated PMMA needs to be separated further before detachment compared to PMMA surfaces coated with C-P55. It is not clear if hydrogen bonds formed between the carbohydrate units on the opposing surfaces affect the disentanglement process. Although the polar groups in the carbohydrate units are hydrated with water molecules, it is conceivable that when the layers come into contact water-carbohydrate hydrogen bonds are partly replaced by hydrogen bonds between carbohydrate units with water being released from the hydration shell.

4.4. The Effect of Carbohydrate Composition on Friction Forces

The friction forces between both C-P55-coated and C-PSLex-coated PMMA are small (see Figure 5) due to exposure of highly hydrated carbohydrate side chains. The higher friction force generated between C-P55-coated PMMA at low loads and the lower friction force between such surfaces at high loads compared to that between C-PSLex-coated PMMA should be due to the difference in carbohydrate composition of these two mucins. The carbohydrate side chains of C-PSLex are longer and more branched than those of C-P55, and thus the number of water molecules accumulated around the carbohydrate chains should also be larger for C-PSLex than for C-P55. Thus, we propose that the lower friction force observed for C-PSLex-coated PMMA at low loads is a result of higher hydration and relatively lower interpenetration
compared to C-P55-coated PMMA. Limited interpenetration and hydration lubrication arising from bound water surrounding the highly hydrated polar groups in the oligosaccharides have also previously been regarded as the reasons for the low friction between sliding mucin layers\textsuperscript{18} and lubricin layers\textsuperscript{40}. In particular, it has been observed that the lubricity decreases after partly or completely removal of the carbohydrate side chains in the mucin domains, which results in reduced hydration.\textsuperscript{46, 47}

Interestingly, the friction force increases more rapidly with applied load for C-PSLex-coated PMMA than for C-P55-coated PMMA. We interpret this as being a consequence of increased chain interpenetration. Just like the longer oligosaccharide chains of C-PSLex counteracted disentanglement during separation and gave rise to a more long-range attraction (Figures 4 b, d, f), it will also counteract disentanglement during sliding. This effect will become more important at higher loads and rationalizes the higher friction force for C-PSLex-coated PMMA than for C-P55-coated PMMA at high loads. Entanglement may also cause whole molecules to be dragged along the surface, which would add another energy dissipative mechanism.\textsuperscript{44, 45}

Again, the possible importance of intrachain hydrogen bonds is an open question, but we note that such bonds have been invoked to explain the transition observed in the friction-load curve obtained for poly(L-lysine)-g-dextran layers where formation and disruption of new hydrogen bonds between interpenetrating chains would dissipate energy.\textsuperscript{41}

5. CONCLUSIONS

We have performed a detailed analysis of the oligosaccharide side chains of two brush-with-anchor type recombinant mucins, C-PSLex and C-P55, and determined their interfacial properties on PMMA using QCM-D and colloid probe AFM. Although both mucin layers have a similar mass (including trapped water), we find obvious differences in their friction-load curves, which we relate to differences in the oligosaccharide side chains. In particular, C-PSLex has longer oligosaccharide side chains than C-P55. In accordance with Amontons’ first
rule, the friction force between C-P55-coated PMMA increases roughly linearly with load after the layers have come in close contact. In contrast, the friction force increases more than linearly with load for C-PSLex-coated PMMA, and thus this system does not follow Amontons’ first rule. At low loads the mucin with longer side chains, i.e. C-PSLex, provides lower friction forces than the mucin with shorter side chains (C-P55). We interpret this as being due to higher hydration and lower chain interpretation for C-PSLex. However, at higher loads, where the chain interpenetration is increased, the situation reverses and now C-P55 provides lower friction forces than C-PSLex. During sliding chain disentanglement must occur, and our data suggest that this causes larger energy dissipation for the mucin with longer carbohydrate side chains (C-PSLex), and this effect becomes dominant at high loads. The observation that the attraction observed during separation of C-PSLex-coated PMMA is of longer range than for C-P55-coated PMMA is interpreted in a similar manner, i.e. chain disentanglement is counteracted by longer oligosaccharide side chains.

ASSOCIATED CONTENT

Supporting Information Available. The method for sialic acid analysis, the molecular structures of the sugar units in the oligosaccharide side chains, the Voigt modelling parameter settings and the quality of the fitting, as well as the tables of putative structures of both O-glycans and N-glycans on C-P55, C-PSLex and BSM (only O-glycans) and their relative amount. This material is available free of charge via the Internet at http://pubs.acs.org.

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