Chemical Biology

Membrane-bound mucins of the airway mucosal surfaces are densely decorated with keratan sulfate: revisiting their role in the Lung's innate defense

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Airway Defense/Glycocalyx/Keratan Sulfate/Membrane Mucin/Periciliary Layer

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Abstract

Understanding the basic elements of the airway mucosal surfaces and how they form a functional barrier is essential in understanding disease initiation, progression, pathogenesis and ultimately treating chronic lung diseases. Using primary airway epithelial cell cultures, atomic force microscopy (AFM), multiangle light scattering and quartz crystal micro balance with dissipation monitoring techniques, here we report that the membrane bound mucins (MBMs) found in the periciliary layer (PCL) of the airway surface are densely decorated with keratan sulfate (KS). AFM and immunoblotting show that the KS sidechains can be removed enzymatically with keratanase II (KII) treatment, and the antibody accessibility for B2729 (MUC1), MUCH4 (MUC4) and OC125 (MUC16) was substantially enhanced. Light scattering analysis confirmed that KII treatment removed ~40% of the mass from the mucin fractions. Surface binding experiments indicated that MBMs were able to pack into a tighter conformation following KS removal, suggesting that negatively charged KS sidechains play a role in mucin–mucin repulsion and contribute to “space filling” in the PCL. We also observed that soluble filtrate from the common airway pathogen *Pseudomonas aeruginosa* is capable of stripping KS from MBMs. Altogether, our findings indicate that KS glycosylation of MBMs may play an important role in the integrity of the airway mucosal barrier and its compromise in disease.

Key words: airway defense, keratan sulfate, membrane tethered mucins, periciliary layer

Introduction

Dysfunction of the airway mucosal barrier, an essential part of the innate immune defense, plays a critical role in the pathology of chronic inflammatory and obstructive airway diseases (Henderson et al. 2014; Ramos et al. 2014; Anderson et al. 2015; Livraghi-Buttrico et al. 2017). Thus, understanding the elements of the airway mucosa and how they interact to form a functional barrier is crucial in understanding and ultimately treating airway obstructive diseases.

Recent studies have modeled the airway mucosal barrier as a two-gel system: an overlying mucus gel composed of the secreted mucins and 100 s of innate defense proteins (Travis et al. 2001; Kesimer et al. 2009), and an underlying periciliary layer (PCL) gel consisting of membrane bound mucins (MBMs), as well as glycosaminoglycans (GAGs) and other glycoconjugates found at the cell and ciliary surface (Stonebraker et al. 2004; Monzon et al. 2006; Kesimer et al. 2013). Limited studies have investigated the relationship between the

properties of the overlying mucus gel and the failure of the mucosal barrier, specifically investigating mucin concentration (Livraghi-Buttrico et al. 2017; Hill et al. 2018; Esther et al. 2019), mucus composition (Sheehan et al. 1995; Radicioni et al. 2016), mucus rheology (Litt 1970; Meyer and Silberberg 1980; Puchelle et al. 1980; King et al. 1997; Lai et al. 2009), mucin ratio (Kirkham et al. 2002; Kesimer et al. 2017), ionic species and hydration (Tarran 2004; Abdullah et al. 2018). Fewer studies have investigated the barrier properties of the PCL.

The barrier properties of the PCL are determined by the constituent MBMs (MUC1, MUC4, MUC16 and MUC20) (Hatrup and Gendler 2008; Derichs et al. 2011; Kesimer et al. 2013), GAGs [keratan sulfate (KS) and hyaluronic acid] (Monzon et al. 2006) and their structural organization and interactions. These elements form a macromolecular mesh (Stonebraker et al. 2004; Button et al. 2012; Kesimer et al. 2013) that limits penetration into the PCL gel (Button et al. 2012). An interesting consequence of this size exclusion is that it forms a semi-permeable membrane; the gel-forming mucins are excluded from the PCL region, while water and ions can freely transport into the gel. This results in an osmotic pressure difference between the mucus layer and PCL, which is driven by the osmotic moduli of the respective layers. When the osmotic modulus of the mucus layer is higher than that of the PCL, such as with mucus hyperconcentration, the osmotic pressure gradient draws water out of the PCL, which leads to dehydration and PCL collapse (Button et al. 2012) and integration with the overlying mucus gel. Understanding the properties of this macromolecular mesh is essential in understanding both how the PCL functions as a barrier and potential interactions that it may have with the mucus layer.

Although the role of MBMs in the airway surface has been studied (Kim 2012; Kato et al. 2014; Kato et al. 2017) and the presence of KS in the PCL is documented (Stonebraker et al. 2004), the role of KS decoration and where exactly it is located is still not clear. In this study, using human bronchial epithelial (HBE) cell cultures, we present evidence that airway MBMs—MUC1, MUC4 and MUC16—exhibit KS glycosylation. While it is well-established that KS is present in both airway secretions (Monzon et al. 2006) and at the epithelial surface (Kesimer et al. 2013), this is the first report directly linking KS to the MBMs of the airway surface, which extend more than a micron away from the cell membrane. We also demonstrate that MBMs with and without KS show differences in packing density. Finally, we show that extract from *P. aeruginosa*, a pathogen common to airway diseases (Doggett et al. 1971; Hoiby et al. 1977; Murphy et al. 2012), including cystic fibrosis, has the capacity to degrade and remove KS from MBMs. Thus, understanding the MBMs and the role of KS glycosylation; particularly their organization and interactions, are of extreme importance in understanding the barrier properties of the PCL in the context of innate airway defense.

Results

Spiked structures are enriched for MUC16 and KS

In our previous work in which we characterized the airway gel forming mucins and their interactions, the mucin interactome (Radicioni et al. 2016), our atomic force microscope (AFM) data revealed spiked-structures that we could not definitively describe (Figure 1). The structures were similar to mucins: long linear filaments with a height ranging from 0.4 to 1 nm interspersed with larger globular protein regions; however, they featured “spike-like” branches, which were absent in our preparations of purified gel forming

Fig. 1. The HBE Mucin network: AFM image of mucins and mucin-associated proteins from HBE cell culture washings. The mucin network (light brown), with gel-forming and membrane-bound mucins, as well as mucin interacting proteins (white). The inset (bottom) is a magnified section showing mucins with a “spiky” morphology; representative spikes have been denoted with red arrows. Scale bar on the top is 500 nm and on the bottom is 200 nm.

mucins MUC5B and MUC5AC and inconsistent with AFM images of MUC5AC, MUC5B in literature (McMaster et al. 1999; Thornton et al. 2000; Radicioni et al. 2016). In addition, the “spike like” regions of the molecule attached to the adjacent mucin chains and formed bundles with them (Figure 1). To identify the structures, which were present in a sample derived from apical HBE cell culture washings, we used isopycnic centrifugation to isolate/separate the biomolecules as a function of buoyant density. We probed the fractions for antibody reactivity to mucins common to HBE cell culture (MUC5B, MUC5AC and MUC16), as well as reactivity to KS (Figure 2). Additionally, we imaged the fractions using AFM and found that fractions with a buoyant density of 1.55 g/mL were enriched for the spiked structure (Figure 2). These fractions showed high OC125 (MUC16 monoclonal) and 5D4 (KS monoclonal) immunoreactivity. Upon further dilution, we observed single units (molecules) of the structure (Figure 2C). We observed heterogeneity in both the degree of “spikiness” between molecules, as well as the length of the spikes on the same molecule. Spikes extended up to 30 nm away from the protein core (Figure 2C and E). The presence of the spikes despite being prepared in 4 M GuHCl rules out any weak ionic interactions,

Fig. 2. Enrichment and isolation of KS rich “spiky” mucins: representative AFM images of fractions taken from an isopycnic gradient of cell culture washings. (A) Fraction 9 has a mucin network consistent with the networks of gel-forming mucins. (B) Fraction 15 is enriched for “spiky” mucins. Scale bar = 500 nm. (C) An AFM image of an isolated MUC16 molecule in a dilute preparation of a fraction rich in KS. The drawn lines represent cross-sectional slices, which are shown in panel (E). (D) A normalized graph of the antibody reactivity for MUC16 (OC125), MUC5B (EU5B) and KS (5D4) for each fraction of the gradient. Fraction 9, which has both MUC5B and MUC16, but low KS, and Fraction 15, which has a higher amount of MUC16 and KS, but less MUC5B are marked on the plot (E). Cross-sectional profile of AFM image in panel (C). The central protein backbone has the same height (~0.6 nm); however, the length that the glycans extend from the central core varies along the molecule.

and the variability in length suggests that the spikes are comprised of repeating units as opposed to fixed length proteins. We observed a variety of core protein lengths but could not determine if the shorter lengths were smaller intact proteins, or fragments of larger proteins. We chose to focus on the longer proteins, which ranged from 1.2 to 2 μm in length, which agreed with the 1.2–1.5 μm contour length previously observed for MUC16 (Kesimer et al. 2013). Based on the similar lengths, the high MUC16 and KS antibody reactivity, we hypothesized that the long spiky structure was MUC16 with variable lengths of KS glycosylation. We were unable to definitively identify MUC1 or MUC4 with spikes via AFM due to the inability to prove that the shorter proteins were not shorter isoforms or fragments of mucin macromolecules.

Airway MBMs are decorated with KS

To test our hypothesis that MBMs are glycosylated with KS, we prepared our spike-enriched fractions and treated them with both endo-beta-galactosidase (KI) (Figure S1) and keratanase II (KII). We found that treatment with KII removed the “spikes” from our samples as observed in AFM images (Figure 3A and B). Furthermore, to verify the removal of the KS, we performed a western blot after agarose gel electrophoresis. After western blotting, we found that KII treatment diminished 5D4 reactivity and dramatically enhanced the reactivity

of the OC125 antibody for the same concentration of MUC16 (Figure 3C). We also observed that KII treatment increased the reactivity of both the MUC1 monoclonal antibody B2729 (Figure 3C) and the MUC4 polyclonal antibody MUCH4 (Figure S2).

Next, we analyzed the effects of KII treatment on the macro-molecular changes of the mucins decorated with KS using size exclusion chromatography multiangle light scattering (SEC-MALS). After running the samples through a CL2B column for size exclusion, the effluent in the void fraction consisted primarily of the large mucins, while all the globular proteins, including the KII enzyme, eluted in the included volume. We found that KII treatment consistently removed mass from the void fraction (Figure 4A and B), interestingly, with a loss of about 40% of the mass. We suggest that this may be an upper limit of the contribution of KS to the molecular weight of the membrane mucins. The average molecular weight had an apparent increase, but this was not statistically significant.

KS-stripped MBMs form a denser layer

Next, we turned our attention to the effect of KS on the physical properties of the MBMs. For this, we used a quartz crystal microbalance with dissipation (QCM-D) system to measure the layer properties of pooled MBM preparations, both with and without KS. We found that the KS-stripped MBMs formed a denser layer; their steady state layer

Fig. 3. KII treatment removes the spikes and increases the antibody signal for MUC1 and MUC16 in an agarose gel: (A) an AFM image of a KS rich fraction before, and (B) after KII treatment. The spikes are present prior to treatment but removed afterward. Scale bar = 200 nm. (C) Agarose gel probing for reactivity to MUC1 (B2729), MUC16 (OC125) and KS (5D4), respectively. Each pair of lanes originates from the apical washings of HBE cultures derived from unique donors. Odd lanes are controls, while even lanes have been treated with KII. In MUC1 and MUC16, there is both an upward shift in the gel after KII treatment, as well as an increase in intensity. KII treatment effectively removes KS.

had an average mass of 354 ng/cm² compared with the average mass of 159 ng/cm² for the untreated samples (Figure 4C). Additionally, the KS-stripped membranes showed a lower layer height and higher viscosity (not shown).

Pseudomonas aeruginosa filtrate reduces KS antibody signal and increases antibody signal against MBM

To understand if a common airway pathogen associated with respiratory infections could target KS glycosylation and if the KS is N- or O-linked to the core mucin, we treated MBMs with *P. aeruginosa* Filtrate (PsaF) and PNGase-f, respectively. We found that PNGase-f had no effect on the B2729 signal for MUC1 signal and reduced the OC125 signal for MUC16 and 2022 signal for KS (Figure 5A). In the MUC16 blot shown in Figure 5A, this is more apparent when the sample is also treated with KII (which increases the antibody reactivity); however, in other blots from other donors, PNGase-f also created a sharp drop in signal compared with the control. In our PsaF experiments, we also tested trypticase soy broth (TSB), which was the growth medium for the PsaF, as well as PsaF that was inactivated through boiling. PsaF reduced the KS signal, but not to the same extent as KII treatment (Figure 5B). We did not see this reduction

Fig. 4. KII treatment reduces the mass of sample that elutes in the CL2B void fraction but increases the packing density (QCM-D): (A) Differential refractive index (dRI, right y-axis) and molar mass (M, left y-axis) traces of the void fraction for a control (black) and KII treated sample (red). The dRI (and by extension the concentration) is reduced after KII treatment. (B) For 3 samples, KII treatment reduced the mass from 29.36 to 17.69 μ g, which represents a statistically significant difference (* $P = 0.0325$). (C) The Sauerbrey calculated mass per unit area of material deposited on a QCM-D sensor for MBMs and KII-treated MBMs. KII-treated MBMs had a statistically significant different average mass per cm² of 354 ng compared with the value of 159 ng for the untreated MBMs * $P = 0.0462$, $n = 3$.

uniformly across all donors, some donors showed very little reduction in KS signal after PsaF treatment. PsaF treatment also increased the OC125 signal for MUC16; however, the increase in signal seemed to be limited to the lower band (Figure 5B).

Discussion

In this study, we have found that MBMs of the airway—MUC1, MUC4 and MUC16—are decorated with KS side chains, most likely via, O-linked glycosylation. We were able to demonstrate this by showing changes in topography, antibody reactivity and apparent mass changes as a result of KII treatment. Although we consistently observed KS, and its subsequent removal with KII across our samples, heterogeneity existed in both the distribution and amount of KS. Although the differences in the amount of KS and individual types of MBMs can be attributed to variations in the amounts produced/harvested from our cell cultures, we did find it interesting that we saw differences in KS distribution; in some donor cultures, KII treatment created a larger increase in MUC1 (B2729) signal; in others, the largest change was in MUC16 (OC125) signal. This could be indicative of donor to donor differences in the ratio of MBM species, and both the total amount and distribution of KS among the MBMs.

Immunoblotting data yielded novel observations; first removing the KS from isolated MBMs, increased the signal for the OC125 antibody for MUC16 as well as the B2729 antibody for MUC1. In the case of MUC16, prior studies have determined that the epitope for OC125, which lies in a tandem repeat region, is independent of glycosylation. OC125 reactivity was present in the absence of

Fig. 5. PNGase-f and PsalF treatment of MBM samples: **(A)** An agarose gel probing for MUC1 (B2729) and MUC16 (OC125) and KS (2022) after treating an MBM sample with PBS (lane 1), PNGase-f (lane 2), KII (lane 3), or PNGase and KII. PNGase-f had little effect on the MUC1 antibody signal but did diminish the MUC16 signal. **(B)** An agarose gel probing for KS (2022) and MUC16 (OC125) after treating MBM samples with PBS (lane 1), KII (lane 2), TSB (lane 3), Boiled PsalF (PsalF-In, lane 4) and PsalF (lane 5). PsalF treatment reduces KS signal but to a lesser extent than KII treatment. It also increases OC125 signal, but this is limited to the lower band.

glycosylation (Bressan et al. 2013), as well as with truncated glycans (Marcos-Silva et al. 2014). KS chains, however, would have been absent in the constructs used in both of these studies. We propose that the increased antibody reactivity observed after enzymatically removing KS is due to resolving steric hinderance: the KS chains in native MBMs restrict epitope access and removing those increases access to the epitope on the protein backbone and correspondingly the antibody signal. Following this hypothesis, if the KS chains were N-linked, then treatment with PNGaseF would produce a similar effect as KII. Our results indicate that PNGaseF treatment decreases OC125 antibody reactivity and slightly reduces (but does not remove) the 2022 signal for KS. The fact that most of the 2022 signal remains despite the stripping of N-linked glycans leads us to conclude that the KS chains are predominantly O-linked to the mucin backbone (however, the blot demonstrates that there may be N-linked KS chains as well). As for the rationale behind the decrease in the OC125 signal

following PNGaseF treatment, we do not have an explanation, but this observation has been previously reported (Kui Wong et al. 2003).

Biophysical characterization of the MBMs before and after KII treatment indicates that KS greatly contributes to the mass of the MBMs. Our SEC-MALS data suggests that KS is a major component of MBM glycosylation. By using size exclusion chromatography with a large exclusion size, a CL2B column, the void fraction is dominated by mucins, while globular proteins (including our added enzyme) and other smaller molecules eluted in the included fraction. Treating the samples with KII removed ~40% of the mass of the void fraction.

QCM-D data objectively showed that the KII-treated MBMs formed a layer with more mass as compared with the untreated MBMs. We attribute this to the mucins being able to pack closer together after having removed the highly negative KS side chains. This is supported by the AFM imaging (Figure 3B), which shows a much tighter organization of mucins after KII treatment, but also in the literature. In the cornea, negatively charged KS proteoglycans are thought to maintain the spacing between collagen fibrils (Kao and Liu 2002). Thus, the negative charge of the KS sidechains on MBMs may similarly be important for maintaining the “structure” of the PCL.

Furthermore, we found that this KS glycosylation can be degraded by the product of the common airway pathogen *P. aeruginosa*. It has been previously reported by Jansen et al. that both *Burkholderia cepacia* and *P. aeruginosa* have the capacity to desulfate gel forming mucins (Jansen et al. 1999). The ability of these pathogens to remove sulfation on MBMs has a deeper implications in that Ramphal et al. found that *P. aeruginosa* showed no adhesion to highly acidic mucin fractions [i.e. a higher degree of sialylation and sulfation as compared with other mucin fractions (Ramphal et al. 1989)], and our finding that the MBMs are sulfated via KS could explain why *P. aeruginosa* does not adhere to the apical cell surface during infection (Heiniger et al. 2010). A paper by Lillehoj et al. (2001) does identify an affinity between *P. aeruginosa* and MUC1, however, that study was performed in CHO cells, which may have had resulted in altered glycosylation. Our findings, along with the relevant literature, suggest that KS glycosylation of the MBMs plays a functional role in the barrier properties of the airway. A similar barrier role of KS/MUC1 was described by Aplin et al., where they showed that endometrial MUC1 (the only other report of a mucin having KS glycosylation) exhibited KS glycosylation and that the KS had anti-adhesive properties. They showed that the degree of MUC1 KS glycosylation varied with the menstrual cycle and was greatly reduced prior to embryonic implantation, thus hypothesizing that KS glycosylation acts as a barrier (Aplin et al. 1998).

Here, we propose that airway KS on the MBMs may have a critical functional role in the barrier properties of the PCL and could be an important determinant for viral and bacterial penetration into the glycocalyx layer and subsequent infection. In addition, some viral pathogens use sugars on cell surface glycosaminoglycans as an attachment and subsequent entry point to airway cells (Aquino and Park 2016). Severe acute respiratory syndrome (SARS) corona virus (CoV), for instance, initially attaches to angiotensin-converting enzyme 2 (ACE2) on the airway surface epithelia (Hoffmann et al. 2020), but heparan sulfate (HS) proteoglycans might be required for further attachment and subsequent infection of the airway cells (Milewska et al. 2014). Tracheobronchial epithelia are not so much enriched with HS, rather KS is robustly expressed on the PCL and glycocalyx (Kesimer et al. 2013), which could be another important viral attachment point for viral pathogens including SARS corona viruses.

In conclusion, based on the visual, biochemical and biophysical evidence, we report that MBMs, especially MUC1, MUC4 and MUC16, are highly decorated with KS chains and propose that these chains are an essential element of the innate immune defense of the PCL and larger airway surface. Moreover, their potential functional role as a barrier would have important ramifications for the progression of mucoobstructive diseases, the ability of pathogens, both viral and bacterial to infect the airway, and the ability to efficiently deliver treatments and therapeutics through the PCL and into cells.

Materials and methods

HBE cell culture and secretion collection

HBE primary cells isolated from non-diseased donor lungs were procured from the Cystic Fibrosis Tissue Culture Core, seeded onto transwell inserts and grown as described by Fulcher et al. (2005). In short, cells were fed with ALI media both basally and apically until they reached 100% confluence, at which point they were grown at an air-liquid interface. Once confluent, cells were fed with ALI media basally three times a week and washed with phosphate buffered saline (PBS) once a week. During washings, 200 μ L of PBS was added apically, cells were incubated at 37°C for 30 min and then apical washings were collected (pooled by individual code) and stored at 4°C.

Isolation of MBMs

Cell culture washings, which contained MBMs shed from the epithelial surface, the gel-forming mucins MUC5B and MUC5AC, as well as other proteins (Kesimer et al. 2009), were mixed 1:1 with 8 M guanidine hydrochloride (GuHCl), to reach a final concentration of 4 M GuHCl. The washings were then brought to a density of 1.45 g/mL with cesium chloride and loaded and sealed into a 40-mL ultracentrifuge tube (Beckman Coulter 344326). Tubes were loaded into a Beckman Ti45 angle rotor and centrifuged using an L8-70M Beckman Ultracentrifuge at 40,000 r.p.m. for 60 h at 14°C. Upon completion of the run, 2-mL fractions were unloaded from the top and collected. Fractions were analyzed with immunoblotting (slot blot) and probed for MUC5B (EU5B, a gift from Thornton et al. 2000), MUC16 (OC125, Cell Marque #325 M-16) and KS (5D4, Seikagaku #270427) reactivity. Fractions with strong KS and MUC16 antibody signal and low MUC5B signal (typically fractions 14–16) were pooled and used as an MBM solution. Higher concentration enrichments [used only to show the presence and change in the antibody signal for MUCH4 after KS removal (Figure S2)] were generated by loading cell culture washings into an ultracentrifuge tube (Beckman Coulter 344060). Tubes were loaded into a Beckman SW 41 Ti swing out rotor and centrifuged at 19,000 r.p.m. for an hour and a half. The pellet was recovered, resuspended in PBS and centrifuged again at 25,000 r.p.m. for an hour. Finally, the pellet was retrieved and resuspended in 1 mL of PBS.

Enzyme treatments

For Keratanase II treatment, the MBM solution was first dialyzed into PBS. Next 0.8 mU of Keratanase II (KII, NATE-1618 Creative Enzymes) was added (or an equal volume of PBS as a control) per milliliter of solution and allowed to incubate overnight at 37°C. The same was done for MUC4, but starting with the higher concentration MBM preparation as described in the previous section. For PNGase

treatment, 2 U of PNGase (F4835-50UN Sigma) in PBS were added to 120 μ L of MBM solution. Samples were incubated overnight at 37°C. For Psf preparation, *P. aeruginosa* filtrate was prepared as in Wu et al. (2005); briefly *P. aeruginosa* was grown in TSB for 72 h at 37°C. The media was then centrifuged, and the supernatant was filtered through a 0.45- μ m filter. For the Psf treatment experiments, five 150- μ L aliquots of the MBM solution were prepared and had 30 μ L of one of the following treatments added: PBS, 0.8 mU of KII in PBS, TSB, Psf boiled for 10 min, and Psf. Treatments were incubated for 16 h at 37°C.

Agarose gel/western blot

MBM Samples were denatured through the addition of urea (final concentration 6 M) and mixed with loading dye and reduced with dithiothreitol. Equal volumes (30 μ L) of the samples were then loaded into a 0.7% w/v agarose gel and run at 90 V for 140 min. Proteins were then transferred to nitrocellulose membranes using a vacuum transfer kit, blocked with non-fat milk (M0841 Lab Scientific) and then probed with antibodies: MUC1 (B2729 a gift from Fujirebio Diagnostics Inc., Malvern, PA, USA), MUC4 (MUCH4 an in-house polyclonal antibody raised against the peptide sequence FLNSNS-GLQGLQFYR), MUC5B (EU5B), MUC16 (OC125) and KS (5D4 or 2022, EMD Millipore #MAB2022). Figures 2, 3 and S1 used 5D4 as the KS antibody, while Figures 5 and S2 used 2022. In general, we found that 2022 had greater reactivity as compared with 5D4, which has specificity for highly sulfated KS. Membranes were washed thoroughly with Tris buffered saline + Tween and then probed with secondary antibodies: IRDye 680LT Goat anti-Rabbit (Licor #926–68021) for the polyclonal antibodies, and IRDye 800 CW Goat anti-Mouse (Licor #926–32210) for the monoclonals. Membranes were then scanned using a LICOR Odyssey and processed with the Odyssey software. Lane shift analysis was done using the gel analyzer tool in ImageJ (National Institutes of Health, Bethesda, MD).

Atomic force microscopy

Samples were desalted/buffer exchanged into PBS using a 5-mL G25 HiTrap desalting column. Fifty microliters of 10 mM nickel chloride were pipetted onto a freshly cleaved mica surface for 1 min, at which point the mica was washed several times by pipetting and collecting MilliQ water on the surface of the mica. Thirty microliters of the sample were pipetted onto the mica and after 3 min; the samples were again washed with MilliQ water and then dried using a nitrogen gun. The sample was then imaged in a Cypher microscope (Asylum/Oxford Instruments, Abingdon, UK) with ArrowUHF tips using noncontact AM/FM tapping mode. Image analysis and processing was performed using the software provided by the manufacturer. Height profiles of mucin molecules and side chains were measured using the AFM software but were aligned by matching the highest central peak and plotted using Graphpad PRISM.

SEC-MALS analysis

SEC-MALS analysis was done by performing chromatography on 100 μ L of injected sample using CL2B columns (2 $\times$ 5 mL Hi-trap columns in series) eluted with 0.2 M NaCl with 10 mM EDTA at a flow rate of 500 μ L/s. Column effluent was then run through a DAWN Heleos (Wyatt, Santa Barbara, CA) and Optilab T-rex (Wyatt) to measure molecular weight and refractive index, respectively. Analysis was done using Astra software (Wyatt).

Quartz crystal microbalance with dissipation

Layer properties of MBMs were measured by using a QCMD Explorer system from Nanosciences. To obtain enough material for QCM-D experiments, washings were collected from cell cultures from several different donors and pooled together. Pooled washings were then enriched as detailed in the isopycnic gradient section. Prior to measurements, a GE AKTA Prime Plus was used for gel filtration, using a 110-mL CL2B column. Samples were loaded into the system and eluted at 1 mL/min with PBS. The column effluent was collected in 5-mL fractions, and a UV spectrophotometer was used to determine the protein peak in the void fraction. The fractions containing the protein peak in the void (typically fractions 7 and 8) were pooled together and concentrated by a factor of 5 using an Amicon Ultra 15 Centrifugal filter unit spin. This protocol was used to both change the buffer to PBS, as well as filtered out keratanase enzyme and removed sugars after enzymatic treatment. Samples were deposited onto the QSX-301 gold chip (Nanosciences, Phoenix, AZ) until it reached a steady state, washed with PBS and then analyzed using the Q-tools software. Mass was determined by using the Sauerbrey equation.

Statistical analysis. A paired sample *t*-test was performed for statistical analysis of SEC-MALS data; an independent *t*-test was performed for analysis of QCM-D experiments.

Supplementary data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org/>.

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Conflict of interest statement

The authors declared no conflict of interest.

Authors' contributions

J.C. and M.K. conceived and designed research. J.C. performed experiments and analyzed results. J.C. and M.K. interpreted results and wrote the manuscript.

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