Mining the “glycocode”—exploring the spatial distribution of glycans in gastrointestinal mucin using force spectroscopy

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ABSTRACT Mucins are the main components of the gastrointestinal mucus layer. Mucin glycosylation is critical to most intermolecular and intercellular interactions. However, due to the highly complex and heterogeneous mucin glycan structures, the encoded biological information remains largely encrypted. Here we have developed a methodology based on force spectroscopy to identify biologically accessible glycopeptides in purified porcine gastric mucin (pPGM) and purified porcine jejunal mucin (pPJM). The binding specificity of lectins Ricinus communis agglutinin I (RCA), peanut (Arachis hypogaea) agglutinin (PNA), Maackia amurensis lectin II (MALII), and Ulex europaeus agglutinin I (UEA) was utilized in force spectroscopy measurements to quantify the affinity and spatial distribution of their cognate sugars at the molecular scale. Binding energy of 4, 1.6, and 26 aJ was determined on pPGM for RCA, PNA, and UEA. Binding was abolished by competition with free ligands, demonstrating the validity of the affinity data. The distributions of the nearest binding site separations estimated the number of binding sites in a 200-nm mucin segment to be 4 for RCA, PNA, and UEA, and 1.8 for MALII. Binding site separations were affected by partial defucosylation of pPGM. Furthermore, we showed that this new approach can resolve differences between gastric and jejunum mucins.—Gunning, A. P., Kirby, A. R., Fuell, C., Pin, C., Tailford L. E., Juge, N. Mining the “glycocode”—exploring the spatial distribution of glycans in gastrointestinal mucin using force spectroscopy. FASEB J. 27, 2342–2354 (2013). www.fasebj.org

Abbreviations: AFM, atomic force microscopy; Fuc, fucose; Gal, galactose; GalNac, N-acetylgalactosamine; GC-MS, gas chromatography–mass spectrometry; GlcNAC, N-acetylgalactosamine; MALDI-TOF, matrix-assisted laser desorption/ionization–time of flight; MALII, Maackia amurensis lectin II; Man, mannose; MTS, mercaptopropyltrimethoxy silane; PBS, phosphate-buffered saline; PEG-3000, O[O-N(3-maleimidopropionyl)aminoethyl]-O’-[3-(N-succinimidylidyloxy)-3-oxopropyl] heptacosahexylene glycol; PGM, porcine gastric mucin; PJM, porcine jejunal mucin; PNA, peanut (Arachis hypogaea) agglutinin; pPGM, purified porcine gastric mucin; pPJM, purified porcine jejunal mucin; RCA, Ricinus communis agglutinin I; TFA, trifluoroacetic acid; UEA, Ulex europaeus agglutinin I

Key Words: atomic force microscopy · lectin-carbohydrate interaction · adhesion · molecular recognition

The mucosal surface of the gastrointestinal (GI) tract is covered by a layer of mucus that ranges in thickness from 300 μm in the stomach to 700 μm in the intestine, and represents the front-line defense barrier between the external environment and the host (1). The mucus layer is structurally organized into a loosely adherent layer facing the lumen and a layer firmly attached to the epithelium surface. The inner mucus layer does not allow bacteria to penetrate, thus keeping the epithelial cell surface virtually free from bacteria, whereas the outer mucus layer is the habitat of the commensal flora (2).

Mucins represent the main structural components of mucus. These large glycoproteins are divided into secreted and cell-surface-bound mucins. The gel-forming mucins contain N- and C-terminal cysteine-rich domains, which are involved in homooligomerization mediated by intermolecular disulfide bonds, which, in turn, results in weblike supramolecular structures critical for the rheological properties of the mucus gel (3). In humans, there are 4 gel-forming mucin genes that are known to be expressed: MUC2 in the intestine, MUC5AC in lungs and stomach, MUC5B in lungs and saliva, and MUC6 in the stomach (1). The cell surface mucins (e.g., MUC1, MUC3A/B, MUC4, MUC12, and MUC13) are expressed on the apical membrane of all mucosal epithelial cells, representing major constituents of the glyocalyx (4). Mucins have a high molecular mass (5×10^5 to 30×10^6 kDa) and >50% (often

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70–80%) of the mucin molecular mass is composed of carbohydrate. These carbohydrate chains are often clustered into highly glycosylated domains, giving the mucin a bottle-brush appearance. In secreted mucins, these O-linked oligosaccharides are concentrated in large peptide variable number of tandem repeat (VNTR) domains of repeating amino acid sequences rich in proline, threonine, and serine (PTS) domains (1). The oligosaccharide content and carbohydrate structures of mucins vary according to cell lineage, tissue location, and developmental stage (5, 6). O-linked glycans contain 1–20 residues, which occur both as linear and branched structures. The carbohydrate chain is initiated with a N-acetylgalactosamine (GalNAc) residue linked to serine or threonine, to which galactose (Gal) or N-acetylglucosamine (GlcNAc) are added to build the core structures, followed by elongation with fucose (Fuc), Gal, GalNAc, GlcNAc residues, and terminated with sialic acid [N-acetylgalactosamine acid (NeuAc)]; or sulfated oligosaccharide species. Alterations in mucin expression, secretion, and glycosylation are commonly observed in inflammatory bowel diseases like Crohn’s disease and ulcerative colitis and colon cancer (7).

The high degree of diversity of the mucin oligosaccharide chains provides many potential binding sites and metabolic substrates for bacteria, and is believed to be an important determinant in the site-specific colonization of commensal bacteria along the GI tract. Furthermore, mucins are likely to be the first molecules that invading microorganisms interact with at the cell surface and thus can limit pathogen binding to other glycoproteins and limit infection (8). Gut bacteria (commensals and pathogens) have evolved adhesins for the ligands that are found in the mucus layers (9, 10). For these reasons, the molecular mechanisms underlying the binding of gut bacteria to carbohydrate structures in mucins is receiving increasing attention.

Atomic force microscopy (AFM) provides a unique combination of high-resolution imaging of minimally treated biological specimens with the ability to probe force with extraordinary sensitivity (11); this combination means that forces can be explored in conjunction with very high spatial resolution. By functionalizing AFM tips with biological molecules, this combined sensitivity can be employed to explore the forces that govern the interaction between receptors and ligands both in vitro (12) and, in certain cases, directly on cell surfaces under physiological conditions (13, 14). Such analysis can yield not just individual values for the rupture force but also detailed information on the energy landscape of the interactions. It is increasingly being acknowledged that complex carbohydrates mediate a huge variety of cellular interactions, permitting and regulating recognition and signaling events. This is achieved through the enormous range and complexity of the branched structures in glycoconjugates and the ability of carbohydrate-binding proteins (lectins) to decipher this “glycocode.” In this report we present a new method based on force spectroscopy to facilitate decoding information present in highly glycosylated mucins.

### MATERIALS AND METHODS

#### Materials

*Ulex europaeus* agglutinin I (UEA), *Ricinus communis* agglutinin I (RCA), peanut (*Arachis hypogaea*) agglutinin (PNA), and *Maackia amurensis* lectin II (MALII) were from Vector Laboratories (Peterborough, UK). Gal, GalNAc, GlcNAc, Fuc, NeuAc, and mucin from porcine stomach [porcine gastric mucin (PGM) type III] were from Sigma Chemical Co. (St. Louis, MO, USA).

#### Mucin preparation

Sigma PGM was purified using a modified method originally developed by Miller and Hoskins (15). The commercial mucin was dissolved by stirring in Dulbecco’s phosphate-buffered saline (PBS) for 1 h at room temperature (21°C). The pH was titrated back to pH 7.4 if necessary using a few drops of 2 M NaOH, and the sample was stirred overnight at room temperature. Any insoluble impurities were removed by centrifugation (10,000 g at 4°C). The mucin was further purified by sequential precipitation in ice-cold ethanol, and the pellet was dialyzed against water and freeze-dried. The purified PGM (pPGM) was dialyzed against a 1 M NaCl solution (16 h at 21°C, 50 kDa molecular mass cutoff; SpectraPore7; VWR International, Lutterworth, UK) prior to AFM studies. For the fucosidase treatment, pPGM (10 mg/ml) was incubated with either 40 or 100 U α1–2 fucosidase (New England BioLabs Inc., Ipswich, MA, USA) in G4 (proprietary) buffer for 24 h at 37°C, without the addition of BSA to avoid BSA binding to the mucin chains. The enzyme was removed from the mucin by gel filtration using a superose 6 HR 10/30 column (GE Healthcare, Little Chalfont, UK) at 1 ml/min as the eluent. The efficacy of the fucosidase treatment was estimated by measuring Fuc release from mucin using the 1-Fuc kit according to manufacturer’s instructions (Megazyme International Ireland, Bray, Ireland). The purified porcine jejunal mucin (pPJM) was obtained from fresh porcine small intestine following previously published purification method (16).

#### Analysis of mucin carbohydrate composition

For the monosaccharide analysis, the glycan antennas were hydrolyzed with trifluoroacetic acid (TFA) and derivatized into deuterated alditol acetates, as described previously (17, 18). For quantification, myoinositol was used as an internal standard for gas chromatography–mass spectrometry (GC-MS). GC-MS analysis was performed using a Thermo Trace MSPlus GC-MS unit with Xcalibur software (Thermo Fisher Scientific Inc., Waltham, MA, USA). The monosaccharide derivatives were separated using a ZB-5MS column (30 m × 0.25 mm × 0.25 μm; Phenomenex, Macclesfield, UK) with helium as the carrier gas at 1 ml/min. The injection of a 1-μl sample was made at 110°C, run for 2 min, followed by an increase to 320°C at a rate of 6°C/min, and finished by a run for 10 min at 320°C. The instrument was used in a split mode with a carrier gas flow rate of 15 ml/min and injector temperature of 200°C. MS data were obtained using the instrument in EI mode with a scan time of 0.4 s for a mass range of 50–700 nm. The GC-MS data were analyzed using
ACD/SpecManager 10.02 (Advanced Chemistry Development Inc., Toronto, ON, Canada). The permethylation for matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) total mass as well as MALDI-LIFT-TOF/TOF sequencing were performed according to Oxley et al. (18) with modifications described in Khoo and Yu (19) for the analysis of the glyccan sulfation. The analysis was carried out on a Ultraflex MALDI-TOF/TOF mass spectrometer (BrukerDaltonics Ltd, Coventry, UK) in both positive and negative ion mode using a nitrogen laser (λ = 357 nm). Samples were cocrystallized 1:1 on a stainless steel target with a saturated solution of 2,5-dihydroxybenzoic acid in 30% acetonitrile and 0.1% TFA. Analysis of the MALDI-TOF data was performed using GlycoWorkbench 2.0 build 83 (20, 21).

The concentration of NeuAc in the samples was determined with the method published by Yao et al. (22). Briefly, 0.33 ml of acidic ninhydrin was mixed with 0.33 ml glacial acetic acid and 0.33 ml of aqueous sample solution, boiled for 10 min in a water bath, and cooled, and the OD was measured at λ = 470 nm. Concentration of NeuAc in the sample was calculated against a calibration curve for NeuAc.

AFM

The atomic force microscope used in this study was an MFP-3D BIO (Asylum Research, Goleta, CA, USA) and it was operated in air using AC mode for imaging. The cantilevers used were Olympus AC160TS (Olympus, Tokyo, Japan) with a nominal spring constant of ~42 N/m, oscillated at a frequency 10% below resonance (typically around 320 kHz). The damping set point for imaging was kept to the minimum value that allowed stable tracking of the sample surface in order to minimize any sample deformation. Images were acquired at a scan rate of 1 Hz. Force spectroscopy experiments were conducted using DC mode under liquid using different cantilevers as described below.

AFM imaging of mucin and lectin-mucin complexes

Immediately prior to imaging, pPGM solutions dialyzed against 1 M NaCl were diluted into ultrapure water to a concentration of 1.5 μg/ml. Deposition was carried out by spotting 4 μl of the diluted sample onto freshly cleaved mica, incubating for 1 min to allow adsorption to take place, and then rinsing the excess liquid off using argon. For lectin-mucin complexes, the dialyzed pPGM (3 mg/ml) was diluted to 1 mg/ml in PBS, and lectins (1 mg/ml in PBS) were mixed in a 1:9.4 ratio and incubated for 40 min at 21°C. Deposition of the complexes onto mica was carried out as above.

Immobilization of lectins on AFM tips

Silicon nitride AFM tips (PNP-TR; NanoWorld AG, Neuchâtel, Switzerland) were functionalized using a 4-step procedure (carried out at 21°C). The first step involved incubation of the tips in a 2% solution of 3-mercaptopropyltrimethoxysilane (MTS; Sigma) in dried toluene (4 Å molecular sieve), followed by washing with toluene and then methanol. In the second step, the silanized tips were incubated for 1 h in a 0.1% solution of O-(N-(3-maleimidopropionyl)aminoethyl)-O’-[3-(N-succinimidylxylo)-3-oxopropyl] heptacosaoxyethylene glycol (PEG-3000; Sigma) in methanol. Unbound PEG-3000 was washed off with methanol, and the tips were dried with argon. The third step involved covalent attachment of lectins by incubation of the tips in 1 mg/ml solutions of the lectin in PBS at pH 7.4 for 1 h at 21°C, followed by a PBS washing step. The fourth step involved incubation of the lectin-functionalized cantilevers in a 10 mg/ml solution of glycine in PBS to amine-cap any unreacted succinimide groups, followed by washing in PBS. Lectin-functionalized tips were stored under PBS at 4°C overnight before use.

Attachment of mucin onto glass slides

The functionalization of clean glass slides with mucin followed the same procedure as for the AFM tips, namely initial silanization with MTS, followed by attachment of the heterobifunctional PEG-3000 linker molecule. A drop (100 μl) of the 1 M NaCl dialyzed mucin (diluted to 1 mg/ml in PBS) was placed onto the derivatized slides for 1 h at room temperature to link the mucin chains via their N termini, followed by washing in PBS. The surfaces were incubated in a 10 mg/ml solution of glycine to amine-cap any unreacted succinimide groups on the glass, and then washed in PBS to remove any unbound glycine, before being inserted into the liquid cell of the AFM.

Force spectroscopy measurements

All binding measurements on mucin-coated glass surfaces were carried out under PBS. For the inhibition studies, a solution of the relevant cognate sugar in PBS was added into the liquid cell to a concentration of 2 mg/ml. The experimental data were captured in a so-called force-volume mode (at a rate of 2 μm/s in the Z direction) and at a scan rate of 1 Hz and a pixel density of 32x32. In this mode, the instrument ramps the Z piezo element of the scanner by a predetermined amount at each sample point over a selected scan area (3 μm) and records the subsequent deflection of the cantilever as it is pushed into (maximum load force 200 pN), then retracted away from the sample surface. This produces a matrix of 1024 force vs. distance curves for each tip-sample combination. The spring constant, k, of the cantilevers was determined by fitting the thermal noise spectra (29), yielding typical values in the range 0.01–0.04 N/m.

Analysis of force-distance curves

Force vs. distance data were analyzed using a bespoke Excel (Microsoft, Redmond, WA, USA) macro (14). The interaction energy was calculated by fitting a straight line to the “off” region of the retraction curve and then calculating the total area associated with any adhesion peaks, which appeared after the AFM tip had broken contact with the glass surface. This approach ensured that any nonspecific tip-sample interactions (which appear at the tip-glass detachment point) were eliminated from the measurements. The separation distances between individual adhesive events in the force spectra were also determined using the macro. Peak identification in the retraction data was carried out by identifying turning points, and discrimination from noise was achieved through the use of a user-adjustable value, which set the threshold level in terms of a multiple of the amplitude of the noise level in the data.

Statistical analysis

The approximation of the theoretical distribution of the nearest binding site separations and of the number of binding sites on the mucin chain by a γ distribution is fully described in Supplemental Data.

The γ probability density function was fitted to the RCA, PNA, UEA, and MALII datasets obtained on standard (pPGM and pPJM) and partially defucosylated pPGM by a nonlinear regression technique. The goodness of fit was evaluated by a χ² test.
The comparison of the distributions of the nearest binding site separations is based on $F$ tests applied to investigate whether the number of distribution functions could be reduced without increasing significantly the error of the fit (24). Those observed distributions that could be fitted by the same $\gamma$ function were considered not to be significantly different.

RESULTS

Imaging mucin chains and lectin-carbohydrate interactions by AFM

The pPGM molecules were imaged in tapping mode in air. After deposition from a dilute solution (1.5 $\mu$g/ml), pPGM adopts an extended fibrous conformation with contour lengths ranging from tens of nanometers to several microns and heights, which for a cylindrical object equate to diameter, ranging from 0.6 to 0.7 nm (Fig. 1A). These polymers closely resemble the size, diameter, and conformations of those seen in AFM images of other mucins (25–27). However, the AFM images of pPGM appeared to present bright spherical features associated with the mucin chains, which cannot be attributed to an increase in measured height due to the chain crossing over itself (Fig. 1A). The presence of such features would prevent accurate identification of specific binding of globular proteins (e.g., lectins) to the mucin chains. These features were largely absent in images of pPGM samples following extensive dialysis against 1 M NaCl (Fig. 1B), suggesting that they arose from the electrostatic binding of impurities to the pPGM chains; the addition of NaCl appears to break the electrostatic complexes, leaving clean mucin chains. Following this treatment, the majority of the bright spots on the mucin chains can be attributed to crossover points and a doubling of height (Fig. 1B).

The chemical composition of pPGM was first defined in terms of the monosaccharide analysis by GC-MS (Supplemental Table S1). This monosaccharide analysis showed that pPGM contained 9.1% Fuc, 5.4% mannose (Man), 34% Gal, 28.9% GlcNAc, and 22.4% GalNAc in the $N$-glycans and 9.8% Fuc, 17.4% Gal, 32.3% GlcNAc, and 39.7% GalNAc in the $O$-glycans, in agreement with data reported in the literature for PGM (28). Sialic acid species were detected separately using a colorimetric assay, accounting for 1% (w/w). MALDI-LIFT-TOF confirmed that the majority of $O$-linked antennas contained 3–12 monosaccharides of Gal, GlcNAc, and GalNAc, with 1–2 Fuc and/or NeuAc modifications (pPGM glycan structures determined by MALDI-LIFT-TOF are archived at http://www.ifr.ac.uk/SPM/PGM_MALDIToF.pdf). The $N$-linked antennas confirmed in the MALDI-LIFT-TOF analysis were a mixture of high-Man bisected or hybrid structures, of which some were fucosylated and especially larger structures were sialylated (archived pPGM glycan structures).

Imaging of carbohydrate-lectin complexes was carried out using the NaCl-dialyzed pPGM sample and lectins specific for mucin glycans; typical images of the complexes are shown in Fig. 2. The fibrous nature of the main mucin chains was clearly resolved and exhibited a constant height ($\approx$0.7 nm), as indicated by the even coloration along the chain length, whereas the lectin molecules attached to the chains appeared as taller objects ($\approx$3.5 nm), and thus as brighter features (Fig. 2, examples indicated by arrows). The AFM images revealed that RCA, UEA, and PNA appeared bound to the mucin chains (Fig. 2). This is in agreement with their expected binding specificity toward pPGM epitope glycans, Gal (RCA and PNA) and Fuc (UEA) (Table 1). The images also showed a small number of lectin molecules not associated with mucin chains as expected from the stochastic nature of ligand receptor binding where bound and unbound states exist in an equilibrium determined by the kinetics of the system, resulting in some lectins nonspecifically bound to the mica surface. The washing steps allowed removing nonspecific binding of lectins to the mucin chains but not from the mica surface.

![Figure 1](image_url)
Force spectroscopy measurements of mucin-lectin interactions

Force spectroscopy provides a basis for exploring specific intermolecular interactions. Here the technique has been used to quantify the interaction between pPGM and lectins, and to map the distribution of glycans along the mucin sidechains. The experimental setup is illustrated schematically in Fig. 3. The lectins were attached to the AFM tip and the mucin was immobilized onto the glass slides using flexible heterobifunctional PEG linkers, which allowed rotational freedom of the mucin molecules. Attachment of the mucin to the glass slide using amine-binding chemistry favors attachment at the N terminus of the mucin chains because this region is free of glycosylation and thus the most accessible. This enhances the ability of the mucin chains to extend into the PBS solution and thus interact with the lectins. An example force-distance curve is presented in Fig. 4A, illustrating the multiple adhesive events that occurred as a lectin (UEA)-functionalized tip was retracted away from the covalently tethered mucin chains in PBS. The relatively large number of adhesion peaks in the retraction curve, and their nonlinear nature, confirmed that the lectin-functionalized AFM tip has repeatedly attached and detached from the mucin chains, with adhesive forces ranging from 50 to 350 pN, consistent with the presence of multiple binding sites along the mucin molecule. Furthermore, the force spectra exhibited binding interactions over distances (up to 3 μm) corresponding to the maximal observed contour lengths of the mucin chains seen in the AFM images (see Fig. 1), further supporting the attachment of the mucin molecules in the N terminus.

The UEA lectin is a Fuc-binding lectin, and addition of Fuc into the liquid cell led to a drastic decrease in the number of adhesive events (from 66 to 4%; see example curve in Fig. 4B). A series of PBS washes to remove Fuc from the imaging chamber subsequently restored the frequency of interactions to 44%. This confirmed that the adhesive peaks seen in the force spectra were due to specific carbohydrate-lectin binding events. This validation step was carried out for force spectroscopy studies using each of the lectin-functionalized tips.

The adhesive energy of the interaction between the lectin-functionalized tips and mucin was determined by integrating the total area of the adhesion peaks in a given force-distance cycle, to produce a measure of the binding affinity. This allowed determination of the full extent of the series of interactions between a given lectin and mucin for the multiplex spectrum. This approach was applied to every curve in each of the force-volume experiments (n=1024), leading to a range of adhesion energy values that can be presented as scatter plots with the frequency of occurrence on the ordinate axis and the range of adhesion energies on the abscissa (Fig. 5). This approach was also applied to the data obtained following the lectin competition experiments in presence of the relevant cognate sugars. The lectins UEA, RCA, and PNA exhibited varying degrees of interaction with pPGM, displaying a modal value of 26 aJ (min–max±sd: 0–507±70), 4 aJ (2–88±12), 1.6 aJ (0.8–10.2±2), respectively. The competitive effect due to the addition of the cognate sugar showed a drastic decrease in interaction energy (Fig. 5), with most of the curves exhibiting no adhesion, confirming the specificity of the interaction. Accordingly, on addition of Fuc, the
modal value for the UEA lectin dataset (Fig. 5A) decreased to 0.5 aJ (0–8.1 ± 2.4) whereas on addition of Gal, the modal value decreased to 0.3 aJ (0–6.8 ± 1.5) and to 0.2 aJ (0.1–9.4 ± 0.6) for the RCA (Fig. 5B) and PNA (Fig. 5C) dataset, respectively. The NeuAc binding lectin, MALII, yielded binding energy data (not shown) consistent with the relatively low level of NeuAc present in pPGM (Supplemental Table S1).

**Mapping of mucin glycans**

In addition to quantifying binding affinity, a thorough analysis of the force spectra can also reveal information about the spatial distribution of the adhesive interactions, as demonstrated in a recent study examining the distribution of 5-methylcytidine bases on DNA sequences (29). Three example force spectra from RCA lectin probing pPGM are presented in Fig. 6, where each curve was captured at different locations on the mucin-coated glass slide. The red curve displays adhesion events that are extensive; they begin around 30 nm from the origin on the x axis (which represents the point at which the AFM tip detached from the surface of the glass slide in the retraction part of the force-distance cycle) and continue for the majority of the pulling range. The blue curve shows clustering of adhesion peaks over a more limited range, and the black curve has two distinct clusters separated by a relatively large distance (> 200 nm), where no interaction occurred (Fig. 6). To extract the spatial distribution of particular sugars along the mucin chains from such data, measurements were made of the separation between the adhesion peaks in each spectrum. This was applied to a whole set of curves for a given force-volume experiment (n = 1024), producing a range of values that can be categorized by histogramic analysis. The resulting raw data, obtained for UEA, RCA, PNA, and MAL-II lectin-functionalized tips probing both pPGM- and pPJM-coated glass slides in PBS, allowed the determination of a single value for the most frequent adhesion event separation for each lectin, and the associated range (Supplemental Fig. S1). A γ probability density function was used to determine the distribution of nearest binding site separations (30). This function reflects a Poisson process with constant density and takes into account that a bound site carries an exclusion zone that prevents unphysical binding configurations. Figure 7 and the results of the $\chi^2$ test (Table 2) confirmed that this type of distribution fits the experimental data very well. This analysis was further used to compare the datasets obtained for the different lectins, yielding information about the proximity of different sugars to one another. An $F$ test showed that the

![Figure 3. Schematic diagram illustrating the force spectroscopy protocol used in this study. A lectin-functionalized AFM tip is retracted away from the surface of a glass slide coated with tethered mucin molecules. The experiment is carried out under PBS solution. Inset: a more detailed view of the interaction between the lectin and glycan side-chains. Labels A–G represent the sugar composition, but not the sequence. Images are not drawn to scale.](image-url)
distributions of the site separations measured on pPGM with RCA, PNA and UEA were not significantly different from each other (estimated between 40 and 48 nm) but different from that observed with MALII (Table 2 and Fig. 8), where the separation between MALII binding sites was estimated to be 2-fold greater (103 nm) than that measured with the other lectins (Table 2). Compared to pPGM, the observed number of MALII binding sites on pPJM was insufficient to estimate the distribution of the site separations, as indicated by the low density values (Fig. 7H). The typical numbers of binding events for the other lectins generally ranged between 1200–2100 for each set of 1024 curves (Supplemental Fig. S1). In addition, the distribution of the separation between UEA binding sites differed significantly between pPGM and pPJM (48 and 64 nm, respectively, \( P < 0.05 \); Fig. 8 and Table 2). Binding site separations were also affected by partial defucosylation of pPGM treated with fucosidase. Figure 9A illustrates a sequential reduction in the position of the fitted peak of UEA binding untreated pPGM and pPJM treated with 40 and 100 U of fucosidase (48, 41, and 25 nm, respectively). The change observed for the 100-U treatment was the most significant (\( P < 0.05 \)), in agreement with the extent of defucosylation, as enzymatically determined by the measure of fucose released (data not shown). PNA binding showed a significant reduction in the position of the fitted peak (33 nm) for the 100-U defucosylated pPGM compared to that obtained on untreated pPGM (47 nm; Fig. 9B).

Figure 10 shows a comparison of the predicted mass distribution function derived from the \( \gamma \) function with the observed number of binding sites for RCA probing pPGM. A \( \chi^2 \) test confirmed that the predicted and observed distributions were not significantly different (\( P > 0.05 \)). The predicted number of binding sites in a 200-nm mucin segment corresponds to ~4 binding sites for RCA, PNA, and UEA, and 1.8 for MALII on pPGM (Table 2). On pPJM, the value decreased to 3 for UEA and was below the detection limit for MALII. On defucosylated pPGM, the number of binding sites per 200 nm increased to 7.8 and 5.9 for UEA and PNA, respectively.

DISCUSSION

Whole mucin molecules have been previously imaged by AFM, displaying both dumbbell-like and fiber-like structures (25–27, 31). Mucins imaged in the present work showed an extended fibrous conformation with contour lengths ranging from tens of nanometers to several microns. This extended linear conformation is likely to be due to steric repulsion between hydrated carbohydrate groups and between carbohydrate and the peptide backbone (32). Previous AFM studies clearly showed that in absence of the sugar residues, the mucin molecules loose their characteristic extended fiber-like structure (26). The combination of different core structures, chain elongation, branching, linkages, and the variety of peripheral and terminal residues results in a high degree of oligosaccharide heterogeneity, with dozens to hundreds of diverse oligosaccharides densely substituting the mucin protein backbone in a bottle-brush configuration (33). Mucin populations can be characterized by different conformational properties and polymer diameters, both of which can be attributed to differences in the degree and nature of glycosylation (31, 34, 35). Here we have visualized the interaction between mucin and the lectins, RCA, PNA, and UEA, specific for the mucin sugars Gal and Fuc as a prerequisite stage to assess the specificity of the interaction. Although in theory such visualization may also provide a means for mapping sugar residues along the mucin chain (30), obtaining images of mucin-lectin complexes that do not contain inter- or intrachain entanglement is difficult once the mucin has been confined to a flat surface (Fig. 2). This becomes particularly problematic with increasing amounts of lectin, as they can initiate cross-linking of the mucin (36), and such entanglement can prevent the unambiguous spatial mapping of lectin binding along mucin chains. However, imaging remains an important step for assessing the purity and suitability of the mucin sample.

Here we applied force spectroscopy in order to obtain quantitative data on the strength and distribution of the interactions between lectins and the mucin chains. The lectins were attached to the AFM canti-
lever tip, and the mucin was immobilized onto the glass surface, both via covalent linker chemistry. Our novel approach is based on a detailed analysis of the force spectra. There are several advantages to this new methodology. The first one is of practical nature—because we are not imaging, we do not need to immobilize the entire mucin chain. Infact, the binding protocol we use allows for the majority of the mucin (and therefore glycan) chains to freely extend into the solution, so that they are more accessible to the probe molecules (lectins) on the AFM tip (Fig. 3). Improving molecular access is important in order to eliminate the experimental differences between binding kinetics determined in 2D confined systems and solution studies (37–39). Our data clearly showed attachment, detachment, and reattachment of the tip-bound lectins to the mucin chains as the AFM tip was retracted (Fig. 4A). This observation is consistent with the "bind and jump" model previously proposed for lectin interaction with mucin chains (37, 38). The fact that many of the clustered binding events do not return to the zero-force baseline suggests that the lectin does not fully detach from the mucin chain but slides along it during the measurement phase as the PEG tether anchoring the lectin to the AFM tip clearly remains extended. The extensive range of distances over which adhesive events occur (up to 3 μm away from the tip-glass detachment point, Fig. 4A) in the force curves demonstrates that the mucin molecules adopt an extended brush-border conformation when attached to the glass surface. Furthermore, the reduction of adhesion on addition of the cognate sugar (Fig. 4B) and the subsequent recovery of the adhesive interactions after removal of the sugar provides unambiguous evidence that the adhesive peaks detected in the force spectra are due to carbohydrate-lectin binding and not to entanglement of the AFM cantilever-tip assembly.

Another advantage of this new approach comes from the novel mode of analyzing the force spectra, as discussed below. Rather than determining the energy landscape of the lectin-carbohydrate interaction in mucin (25, 37), the first aspect of our analysis was to determine the total binding affinity between the lectins and mucin. We believe that this is relevant to in vivo situations such as the adhesion of bacterial cells to the GI tract mucus layer, which, due to the nature of the bacterial cell surfaces, are likely to be the result of multivalent binding events (40). Hence, knowledge of the distribution and accessibility of the glycans will help advance our understanding of bacterial attachment to the mucin. The Fuc-binding lectin, UEA, showed the strongest affinity for the mucin chains, followed by that

Figure 5. Interaction energy plots obtained using lectin-functionalized AFM tips to probe pPGM in PBS (gray) and following the addition of the relevant cognate sugars (black). A) UEA. B) RCA. C) PNA.

Figure 6. Example force spectra obtained by probing a pPGM-coated glass slide at 3 discrete locations with an RCA-functionalized AFM tip in PBS. Arbitrary offsets in the y axis have been added to the blue and black datasets for clarity of presentation.
of the Gal-binding lectins, RCA and PNA (Fig. 5). The observed difference between RCA and PNA may reflect their binding specificities to Gal. While RCA is specific to terminal Gal, PNA can recognize terminal Gal, $\beta1-3$ Gal, and galactosamine. Both lectins are sensitive to sulfate substitution on the Gal ring. For RCA, binding is enhanced by 2-O- or 6-O-sulfation but abolished by 4-O-sulfation (41). In contrast, the presence of sulfates generally reduces the binding affinity of PNA to terminal Gal (42) whereas or 6-O- or 3-O-sulfation or sialation of the Gal residue in Gal$\beta1,3$GalNAc can almost abolish PNA binding (43). In addition, PNA does not bind to the acetylated form of galactosamine (44). MALDI-TOF analysis in negative ion mode revealed the presence of diverse sulfated structures in pPGM (archived pPGM glycan structures). Thus, the affinity differences seen for RCA and PNA in the present study may arise from the sulfation profile of some of the Gal residues in pPGM.

In addition to the chemical structures of mucin oligosaccharides, the density, distribution pattern, and 3D architecture of the sugar molecules are also relevant for the formation of the glycoepitopes presented to the local environment in the GI tract and, therefore, affect their biological interactions and recognition by gut bacteria. The variation in the captured adhesion pro-
files from the force spectroscopy data can inform on the structural heterogeneity that exists within a population of mucin molecules (Fig. 6). Strikingly, the adhesion peaks tend to occur in clusters, which may suggest a block-like distribution of the target epitopes along some mucin chains. However, since ligand-receptor binding is a stochastic process, the probabilistic nature of adhesion between the lectin and its target sugar needs to be taken into consideration when mapping is intended. This was addressed by measuring the separation between neighboring adhesive events in each force spectrum and then combining the data from a given experiment (i.e., from 1024 curves) into a single distribution histogram for each lectin (Supplemental Fig. S1) to ensure significance of the statistics derived from the measurements. A similar approach was recently used for the mapping of 5-methylcytidine bases along the mucin main chain (Fig. 3).

Here we showed that the $\gamma$ distribution function is an adequate approximation for the distribution of the nearest binding site separations obtained for the different lectins probing the mucin molecules. Although interchain jumping cannot be excluded for the long-distance binding events seen in the force spectra, it is unlikely to be the case for the smaller separation binding events, which are contained within a given cluster in the force spectra (the discrimination limit was hence set to 200 nm for the $\gamma$ analysis). Once the tip-tethered lectin binds onto a particular mucin chain, the proximity of target sugars available to the lectin will be significantly closer on the bound chain than any of the others unless they were very tightly packed. Following the force acquisition, AFM imaging of the mucin-coated glass slides confirmed that the attachment protocol used did not produce a close-packed monolayer of mucin chains on the glass surface (data not shown). In addition, no steric effects reminiscent of a tightly packed polymer brush (45) were obtained in the contact region of the force curves (Figs. 4 and 6). However, once the lectin has detached for a significant distance (i.e., >200 nm, typified by the featureless regions of the retraction force curve following the occurrence of a cluster), the next cluster of binding events may result from the lectin hopping onto a different mucin chain. The fits to the data shown in Fig. 7 confirmed that binding site separations with high frequencies comprised values up to 200 nm, whereas larger values were detected with very low frequencies. This reinforces the

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**TABLE 2.** Analysis of nearest neighbor binding site separations detected with different lectins

| Lectin | $\chi^2$ | $P$ | Expected separation (nm) | Median | Predicted binding sites ($n/200$ nm) |
|--------|--------|-----|--------------------------|--------|-------------------------------------|
| **On pPGM** |        |     |                          |        |                                     |
| RCA    | 19     | 0.86| 44.16 ± 32.82            | 36.15  | 4.30 ± 1.59                         |
| PNA    | 1      | 0.99| 47.07 ± 33.89            | 39.27  | 3.98 ± 1.46                         |
| UEA    | 8      | 0.50| 47.48 ± 32.26            | 40.35  | 3.94 ± 1.40                         |
| MALII$^*$ | 19     | 0.20| 103.42 ± 93.15           | 77.10  | 1.84 ± 1.06                         |
| **On pPJM** |       |     |                          |        |                                     |
| RCA    | 11     | 0.99| 53.44 ± 41.37            | 43.26  | 3.54 ± 1.48                         |
| PNA    | 13     | 0.98| 40.48 ± 29.41            | 33.75  | 4.70 ± 1.63                         |
| UEA    | 13     | 0.99| 63.99 ± 48.25            | 52.67  | 2.91 ± 1.30                         |
| **On DF-pPGM** |      |     |                          |        |                                     |
| UEA, 40 U | 5     | 0.99| 41.14 ± 32.04            | 33.05  | 4.64 ± 1.64                         |
| UEA, 100 U | 2    | 0.99| 24.58 ± 16.07           | 21.04  | 7.81 ± 1.85                         |
| PNA, 100 U | 14   | 0.97| 32.94 ± 28.30           | 25.18  | 5.93 ± 2.11                         |

Fit of $\gamma$ distribution indicates goodness of fit of the $\gamma$ distribution density function. Expected separation and predicted binding site data are presented as means ± s.e. DF-pPGM, partially defucosylated porcine gastric mucin. $^*$Insufficient data to fit to pPJM set.
assertion that the majority of the data used to derive the information provided in Table 2 corresponds to binding sites located on the same mucin molecule. Therefore, since the γ distribution function is a good approximation for the distribution of binding site separations (Fig. 7), the distribution of the number of binding sites in the 200 nm of the mucin molecule can be predicted by mathematical modeling (Fig. 10) following the protocol as detailed in Supplemental Data. Another important safeguard when performing this analysis is to examine the time domain in which the information is gathered to ensure that it remains below the inherent resonance of the AFM cantilever-tip assembly. The cantilevers used in this study have a fundamental resonance centered on 2.1 kHz when immersed in the buffer solution, and a series of higher Eigen modes. In this work, the frequency of the modal separation data, calculated from the reciprocal of the distance divided by the z piezo velocity of the measurements, equates to 117 Hz for the mode, which is ~5% of the cantilever fundamental resonance. Using this approach, we showed that the entire range of separation values captured was significantly below the cantilever resonance peak.

Having determined the spatial distribution of particular sugars along the mucin main chain, it now becomes possible to begin to tease out details of the glycan chain composition by comparing the distribution profiles obtained with the different lectins (Fig. 8). The RCA, PNA, and UEA datasets showed similar profiles (Fig. 8), whereas the separation between binding sites derived from the MALII dataset on pPGM was 2-fold greater (Table 2), as expected from the mucin chemical structure, where NeuAc represents only 1% of the total monosaccharide content (Supplemental Table S1 and ref. 28). In addition, the distribution of the separation between binding sites for UEA was significantly different between untreated and partially defucosylated pPGM, and the modal value varied in a systematic manner correlated to the extent of the enzymatic treatment (Fig. 9A). Intriguingly, the separation value between UEA binding sites was reduced with removal of Fuc. Probing of the defucosylated mucin sample with PNA (which targets Gal) revealed a similar trend (Fig. 9B), suggesting that partial removal of Fuc (which is found in numerous positions in both N- and O-glycan epitopes, as detected by MALDI-LIFT-TOF, archived pPGM glycan structures) reduces the spacing between neighboring glycan antennas due to debranching of the antennas. Thus the method of analysis presented here reveals not only quantitative information about the distribution of glycans along the mucin main chain, but the relative comparisons of the distributions can also provide detail of the composition of the glycan chains themselves (i.e., the nature but not the sequence of A–G, using the analogy in Fig. 3). In addition to lectins, antibodies and carbohydrate binding modules (CBMs) of carbohydrate active enzymes could also be used to probe the mucin molecules using the proposed methodology. Since these proteins possess a large range of unique specificities, collation and comparison of such data will advance our knowledge on the glycosylation pattern of mucins and the accessibility of the glycopeptidic to binding. With the data generated by this new method, we can begin to decipher the mucin glycome used by members of the microbiota. This approach opens up future possibilities for developing mathematical models to predict how these glycans contribute to the selection of bacterial species leading to homeostasis or dysbiosis and disease.

Figure 9. Comparison of the γ density distribution function fitted to the observed binding site separations. A) UEA-functionalized AFM tip probing: green trace, pPGM; dark brown trace, 40 U fucosidase-treated pPGM; light brown trace, 100 U fucosidase-treated pPGM. B) PNA-functionalized AFM tip probing: blue trace, pPGM; light brown trace, 100 U fucosidase-treated pPGM. Same letter indicates distributions that are not significantly different according to an F test.

Figure 10. Observed frequencies (black) and predicted mass function (gray) for the number of binding sites in a mucin segment of 200 nm length for RCA-functionalized AFM tips probing pPGM.
CONCLUSIONS

We have demonstrated that force spectroscopy can be used to characterize the distribution of specific carbohydrate species and reveal differences in the highly complex structures of different mucins. Comparison of the spatial information derived from mucin probed with different lectins provided additional information about the composition of the side chains. This new approach is relevant to biological interactions, which mediate host-bacterial interactions and disease pathology in the GI tract. In addition, this method provides the ability to quantify the binding affinity between carbohydrate moieties and lectins and so provides a blueprint to measure the molecular recognition pattern of bacterial adhesins (from commensals and pathogens) to mucins. Taken together, these approaches will help in deciphering the molecular mechanisms underlying bacteria-mucous interactions in the GI tract.

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