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Mucin–lectin interactions assessed by flow cytometry

Faye Jeffers, Christine Fuell, Louise E. Tailford, Donald A. MacKenzie, Roy J. Bongaerts *, Nathalie Juge *

Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

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ABSTRACT

The O-glycosylated domains of mucins and mucin-type glycoproteins contain 50–80% of carbohydrate and possess expanded conformations. Herein, we describe a flow cytometry (FCM) method for determining the carbohydrate-binding specificities of lectins to mucin. Biotinylated mucin was immobilized on streptavidin-coated beads, and the binding specificities of the major mucin sugar chains, as determined by GC–MS and MALDI-ToF, were monitored using fluorescein-labeled lectins. The specificities of lectins toward specific biotinylated glycans were determined as controls. The advantage of flexibility, multiparametric data acquisition, speed, sensitivity, and high-throughput capability makes flow cytometry a valuable tool to study diverse interactions between glycans and proteins.

Mucins are heavily O-glycosylated glycoproteins that are secreted by higher organisms to protect and lubricate epithelial cell surfaces from biological, chemical, and mechanical insults. Mucin and mucin-type molecules exist as soluble and membrane-attached molecules that are involved in modulating immune response, inflammation, adhesion, and tumorigenesis.[1] The O-glycosylated domains of mucins and mucin-type glycoproteins contain 50–80% of carbohydrate and possess expanded conformations. Polypeptide tandem repeats are found in mucins that contain clusters of Ser and Thr residues in high content.[2] The O-linked carbohydrate chains in these domains are attached via α-GalNAc residues to Ser and Thr residues, which have been shown to induce a threefold expansion of the polypeptide chain of these molecules.[3,4] Mucins have received considerable interest in recent years and several methods have been used for the glycoprofiling of these highly complex molecules. Mass spectrometric (MS) analyses by soft ionization techniques, electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) have been employed in most studies,[5–8] revealing a very large oligosaccharide catalog, with respect to size, diversity, and quantity of specific glycans and terminal epitopes.[9–12] This represents an enormous repertoire of potential binding sites for micro organisms inhabiting these mucosal surfaces and research is underway to investigate the nature and specificity of the bacterial glycan-binding proteins (lectins) involved in this process.

There is a variety of approaches to the determination of detailed sugar-binding specificity of lectins: these include hemagglutination inhibition measurements,[13] enzyme-linked immunosorbent assay (ELISA),[14] enzyme-linked lectin assays (ELLA),[15] surface plasmon resonance (SPR),[16–19] isothermal titration calorimetry (ITC),[13] frontal affinity chromatography (FAC),[20] and affinity chromatography on immobilized lectin columns.[21] However, when the ligands of lectins involved in cellular recognition are sugar moieties attached to proteins and/or lipids and when only limited amounts of material are available; it is often extremely difficult to elucidate the specificities of these interactions in detail at the molecular level. Among the analytical systems developed, glycan microarrays may be the most promising approach for high-throughput investigation of glycan-binding proteins,[22–24] however it is relying on the availability of glycan arrays presenting the right type of sugar. The current mammalian glycan array (version 4.1) developed in the Consortium for Functional Glycomics (CFG, www.functionalglycomics.org) have 465 glycan targets, however although the library size is continually expanding, given the density, microenvironment, context and complexity of the O-glycans present on mucins, assessing binding to the whole mucin molecules is essential.

In this work we used flow cytometry (FCM) as an alternative method to study the interaction between lectins, glycans, and mucin. FCM is a routine method for cellular biology that has been used since the 1970s (for a review, see[25]) and is extensively used in clinical diagnostics and biomedical research. The fast data acquisition speed and quantitative multiparametric capacity of this technology, together with cell/particle sorting options, have made it a valuable tool in environmental microbiology[26,27] and food research.[28,29] In contrast, reports on the use of FCM for protein–glycan interactions are scarce.[30–32] Here, we used the highly purified, weakly charged, short-side-chain, heavily glycosylated mucin, porcine gastric mucin (PGM) similar to human MUC6.[33,34]

* Corresponding authors. Tel.: +44 1603 25500; fax: +44 1603 507723 (R.J.B.); tel.: +44 1603 255068; fax: +44 1603 507723 (N.J.).
E-mail addresses: roy.bongaerts@bbsrc.ac.uk (R.J. Bongaerts), nathalie.juge@bbsrc.ac.uk (N. Juge).

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To establish the chemical structure of PGM, we performed monosaccharide and linkage analyses by GC–MS, whole epitopes analysis after permethylation with MALDI-ToF/MS, and amino acid analysis. The monosaccharide analysis and linkage determination with GC–MS are shown in Table 1. The relative amount of the single monosaccharides has been calculated using their response factors in the GC spectrum in relation to the standard spectrum and myo-inositol as internal standard. Here, the N-glycans and O-glycans were not separated for analysis, in agreement with the presence of mannose. Fucose, galactose, *N*-acetylglucosamine (GlcNAc), and *N*-acetylgalactosamine (GalNAc) were detected as the major monosaccharide species, accounting for over 90% of all the monosaccharide residues (Table 1). Sialic acid species were detected in trace levels using a colorimetric assay. MALDI-ToF confirmed that the majority of O-linked antenna contained three to six monosaccharides of glucose, galactose, GlcNAc, and GalNAc with one to two fucose and/or sialic acid modifications. The overall weight fraction of sugars in the mucin sample is estimated as 71–76% (w/w) (Table S1). This value is in agreement with literature values for sugar/protein ratios for mammalian gastric/colonic mucins.[35–38]

For the flow cytometric analysis, we selected lectins with known specificities to the terminal sugars present in the mucin under investigation (Table S2). The binding of fixed amount of biotinylated mucins or glycans, immobilized on streptavidin beads with varying amounts of FITC (fluorescein isothiocyanate)-labeled lectins was determined by the measure of the fluorescence using FCM. The coupling process and the density of bead-coupled ligands are important for the performance of the assay. The binding capacity of microspheres is 0.12 μg biotin-FITC/mg microspheres and a 10-fold excess amount of glycan/glycoprotein was used in each reaction. The saturation of the bead-binding sites by mucin was confirmed by measuring the protein amount in the supernatant fraction of the coated beads after centrifugation (see Section 1).

Flow cytometric analyses showed specific binding of the lectins to their cognate glycans and mucin, as compared to the binding of lectins to aminoglucitol-coated beads (used as negative control) (Figs. 1 and 2). Figure 1A shows an example of a bivariate scatter plots obtained for WGA against GlcNAc and aminoglucitol, showing a 19-fold difference in median FITC fluorescence between GlcNAc and aminoglucitol-coated beads. The results were highly reproducible with minimum standard deviation even at low lectin concentration range (Fig. 1B; Fig. 2B), which is a considerable advantage compared to other biophysical techniques (for a review, see[39]). Taking into account the fluorescence/protein (F/P) molar ratio of WGA (2.9), UEA (2.8), and RCA (5.3), the binding to mucin was highest for UEA I and WGA and followed by RCA (Fig. 2B), which is in agreement with the presence of terminal fucose and sialic acid sugar epitopes in the mucin used in this study, as determined by GC–MS and MALDI-ToF (Table 1).

Biotinylation of mucins did not affect the recognition by lectins as chemically-functionalized beads (with thiol-terminal silanes

Table 1
Monosaccharide and linkage analyses by GC–MS of mucin glycans

Monosaccharide	% Sugar		Linkages
	Un-treated	De-sialylated	
Fucose (Fuc)	16.8	16.7	*Terminal* (1,5-Di-*O*-acetyl-1-deuterio-6-deoxy-2,3,4-tri-*O*-methyl-L-galactol)
Mannose (Man)	0.8	1.1	*2-Linked* (1,2,5-Tri-*O*-acetyl-1-deuterio-3,4,6-tri-*O*-methyl-D-mannitol) *4-Linked* (1,4,5-Tri-*O*-acetyl-1-deuterio-2,3,6-tri-*O*-methyl-D-mannitol) *3,6-Linked* (1,3,5,6-Tetra-*O*-acetyl-1-deuterio-2,4-di-*O*-methyl-D-mannitol)
Glucose (Glc)	6.8	6.7	*2,3-Linked* (1,2,3,5-Tetra-*O*-acetyl-1-deuterio-4,6-di-*O*-methyl-D-glucitol) *2,6-Linked* (1,2,5,6-Tetra-*O*-acetyl-1-deuterio-3,4-di-*O*-methyl-D-glucitol) *3,6-Linked* (1,3,5,6-Tetra-*O*-acetyl-1-deuterio-2,4-di-*O*-methyl-D-glucitol
Galactose (Gal)	46.5	45.5	*3,6-Linked* (1,3,5,6-Tetra-*O*-acetyl-1-deuterio-2,4-di-*O*-methyl-D-galactitol) *2,3,6-Linked* (1,2,3,5,6-Penta-*O*-acetyl-1-deuterio-4-*O*-methyl-D-galactitol) *Terminal* (only in desialylated) (1,5-Di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methyl-D-galactitol)
GlcNAc	14.5	14.5	*6-Linked* (1,5,6-Tri-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3,4-di-*O*-methyl-D-glucitol) *4,6-Linked* (1,4,5,6-Tetra-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3-*O*-methyl-D-glucitol) *Terminal* 1,5-Di-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3,4,6-tri-*O*-methyl-D-glucitol
GalNAc	14.5	15.5	*3-Linked* (1,3,5-Tri-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-4,6-di-*O*-methyl-D-galactitol) *4-Linked* (1,4,5-Tri-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3,6-di-*O*-methyl-D-galactitol) *6-Linked* (1,5,6-Tri-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3,4-di-*O*-methyl-D-galactitol) *4,6-Linked* (1,4,5,6-Tetra-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3-*O*-methyl-D-galactitol)
Neu5Ac[a]	0.1	N/D	N/A

N/D = not detectable; N/A = not applicable.
[a] The sialic acid content was estimated with the colorimetric assay (see Section 1), therefore linkage analysis was not performed.