

Identification of high-mannose and multiantennary complex-type *N*-linked glycans containing α -galactose epitopes from Nurse shark IgM heavy chain

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Abstract MALDI-TOF mass spectrometry, negative ion nano-electrospray MS/MS and exoglycosidase digestion were used to identify 36 *N*-linked glycans from 19S IgM heavy chain derived from the nurse shark (*Ginglymostoma cirratum*). The major glycan was the high-mannose compound, Man₆GlcNAc₂ accompanied by small amounts of Man₅GlcNAc₂, Man₇GlcNAc₂ and Man₈GlcNAc₂. Bi-

and tri-antennary (isomer with a branched 3-antenna) complex-type glycans were also abundant, most contained a bisecting GlcNAc residue (β 1→4-linked to the central mannose) and with varying numbers of α -galactose residues capping the antennae. Small amounts of mono-sialylated glycans were also found. This appears to be the first comprehensive study of glycosylation in this species of animal. The glycosylation pattern has implications for the mechanism of activation of the complement system by nurse shark IgM.

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 α -Galactose

Abbreviations

ABS	<i>Arthrobacter ureafaciens</i> sialidase
BTG	bovine testis β -galactosidase
CBG	Green coffee-bean α -galactosidase
DHB	dihydroxybenzoic acid
dHex	deoxy-hexose
EDTA	ethylenediamine tetra-acetic acid
Gal	galactose
GlcNAc	<i>N</i> -acetylglucosamine
Hex	hexose
HexNAc	<i>N</i> -acetylhexosamine
IgM	immunoglobulin M
MALDI	matrix-assisted laser desorption/ionization
Man	mannose
MBL	mannose-binding lectin
MS	mass spectrometry
PAGE	polyacrylamide gel electrophoresis
PNGase	protein <i>N</i> -glycosidase

Q	quadrupole
SDS	sodium dodecylsulphate
SPH	<i>Streptococcus pneumoniae</i> β - <i>N</i> -acetylhexosaminidase
TOF	time-of-flight

Introduction

Sharks belong to the oldest vertebrate kingdom known to possess an adaptive immune system that uses immunoglobulin (Ig). IgM is one of the three Igs commonly found in this species [1], the other two being IgNAR and IgW [2]. Very little is known about the glycosylation of fish IgM; some early work reported compositions of the glycans from bony fish, namely pike IgM [3] and Atlantic salmon [4] but no structural details were elucidated. A later study on IgM from Atlantic cod [5] revealed a series of bi- and triantennary *N*-linked glycans of the type normally found in mammals. This paper reports the structures of *N*-linked glycans released from 19S IgM heavy chain from the cartilaginous fish, nurse shark (*Ginglymostoma cirratum*), a species whose IgM has seven consensus sequences for *N*-linked glycosylation [6], but for which there appear to be no details of the attached glycans. The structures of the glycans were determined using newly developed mass spectrometry techniques based on matrix-assisted laser desorption time-of-flight and negative ion nanospray MS/MS mass spectrometry [7–9].

Materials and methods

Extraction and preparation of IgM

Blood was obtained from a nurse shark under anaesthesia and was allowed to clot. Serum (15 ml) was diluted with 105 ml water at 4°C and stirred for 100 min at 4°C. The precipitate was harvested by centrifugation at 3500 g for 20 min at 4°C. The supernatant was removed, and the pellet was resuspended in 50 ml of 1.25 mM sodium phosphate, 17.5 mM NaCl, pH 7.4 and centrifuged again. This euglobulin precipitation step was based on that used for carp serum [10]. The pellet was redissolved in 5 ml of 10 mM sodium phosphate, 500 mM NaCl, and mM EDTA at pH 7.4. Oligomeric (19S) IgM was purified from the redissolved pellet by gel filtration on a 50 × 1.6 cm diameter column of Superose 6 (GE Healthcare) equilibrated in Dulbecco's PBS-0.5 mM EDTA, pH 7.4. The presence and purity of IgM was observed by SDS-PAGE analysis [11] using reduced and non-reduced samples [12].

Enzymatic release of *N*-linked glycans

Oligosaccharides were released from the IgM heavy chain with protein *N*-glycanase (PNGase) F (Prozyme, San Leandro, CA, USA) from within Coomassie blue-stained SDS-PAGE gels following the method of Küster *et al.* [13]. Bands containing approximately 10 µg of IgM heavy chain were excised from reducing SDS-PAGE gels and washed with 20 mM NaHCO₃ pH 7.0. The washed gel bands were dried in a vacuum centrifuge before rehydration with 30 µl of 30 mM NaHCO₃ pH 7.0, containing 100 Units/ml of PNGase F. After incubation for 12 h at 37°C, the enzymatically released *N*-linked glycans were eluted with water. Salts were removed by incubation at room temperature (5 min) with 200 µl of an acid-activated AG-50W (200–400 mesh) slurry (BioRad, Hercules, CA, USA), which was removed by filtration with a 0.45 µl pore-size filter (Millex-LH, hydrophobic polytetrafluoroethylene). Residual glycosylamines from the PNGase F-release were converted into glycans by reaction with 1% formic acid for 40 min at room temperature. The extracted glycans were further purified for mass spectrometry by drop dialysis on a Nafion 117 membrane [14]. Glycans were desialylated either by digestion with *Arthobacter ureafaciens* sialidase (see below) or by heating at 80°C with 1% acetic acid for 30 min.

Exoglycosidase digestion

The glycans in 50 mM sodium acetate buffer, pH 5.5 were incubated for 16 h at 37°C with the following enzymes (Glyko Inc Upper Heyford, UK): *A. ureafaciens* sialidase (EC 3.2.1.18, ABS, specificity for α 2→3/6-linked sialic acid); at 1–2 U/ml; a mixture of ABS and bovine testis β -galactosidase (EC 3.2.1.23, BTG, specificity for β 1→3/4-linked galactose), 1 U/ml; a mixture of ABS, BTG and green coffee bean α -galactosidase (EC 3.2.1.22, CBG, specificity for α 1→3/4/6-linked galactose); Jack bean α -mannosidase, (EC 3.2.1.24), 1 U/ml. a mixture of ABS, BTG and *Streptococcus pneumoniae* β -*N*-acetylhexosaminidase, (SPH, specificity for β 1→2-linked GlcNAc at the concentration (0.01 U/ml) used. At this concentration GlcNAc residues linked β 1→2- to mannose on the 6-antenna are not cleaved in the presence of a bisecting GlcNAc). After digestion, enzymes were removed using protein-binding Millipore Micropure-EZ filters (Millipore), the samples were cleaned with a Nafion membrane and examined by MALDI-TOF MS.

MALDI-TOF MS

Positive ion MALDI spectra were obtained with a Waters–Micromass TofSpec 2E time-of-flight (TOF) mass spec-

trometer (Waters MS Technology Ltd, Manchester, UK) operated in reflectron mode. The pulse and acceleration voltages were 3 and 20 kV respectively. Data acquisition and processing were performed with MassLynx software version 3.3. Samples were prepared by mixing an aqueous solution of the glycans (0.5 μ l) with a saturated solution of 2,5-dihydroxybenzoic acid (DHB) in acetonitrile (0.3 μ l) on the stainless steel MALDI target and allowing the mixture to dry under ambient conditions. The dried sample spot was then re-dissolved in ethanol (0.2 μ l) and again allowed to dry.

Electrospray mass spectrometry

Electrospray mass spectrometry was performed with a Waters-Micromass quadrupole-time-of-flight (Q-ToF) Ultima Global instrument in negative ion mode. Samples in 1:1 (v:v) methanol:water were infused through Proxeon nanospray capillaries (Proxeon Biosystems, Odense, Denmark). The ion source conditions were: temperature, 120°C; nitrogen flow 50 L/h; infusion needle potential, 1.2 kV; cone voltage 100 V; RF-1 voltage 150 V. Spectra (2 sec scans) were acquired with a digitization rate of 4 GHz and accumulated until a satisfactory signal:noise ratio had been obtained. For MS/MS data acquisition, the parent ion was selected at low resolution (about 5 m/z mass window) to allow transmission of isotope peaks and fragmented with argon at a pressure (recorded on the instrument's pressure gauge) of 0.5 mBar. The voltage on the collision cell was adjusted with mass and charge to give an even distribution of fragment ions across the mass scale. Typical values were 80–120 V. Other voltages were as recommended by the manufacturer. Instrument control, data acquisition and processing were performed with MassLynx software Version 4.0 (Waters). Glycans were observed as phosphate adducts.

Results

SDS-PAGE analysis of the reduced IgM material showed the two expected bands of apparent molecular weights 65–70 kDa and 25 kDa. The non-reduced material did not enter the 3.5% acrylamide stacking gel (Fig. 1) consistent with the protein being oligomeric, not monomeric, immunoglobulin. N-terminal sequence analysis of the heavy (70 kDa) chain revealed slight heterogeneity (<7%), but with a major sequence EVTLTQPEAENSQPG, which is consistent with a previously-reported sequence EVTLTZPZAZ for *G. cirratum* heavy chain of 19S Ig. [15].

Figure 2 shows the MALDI-TOF spectra of the released glycans. The glycans were predominantly neutral with very little sialic acid capping the antennae; MALDI-TOF and

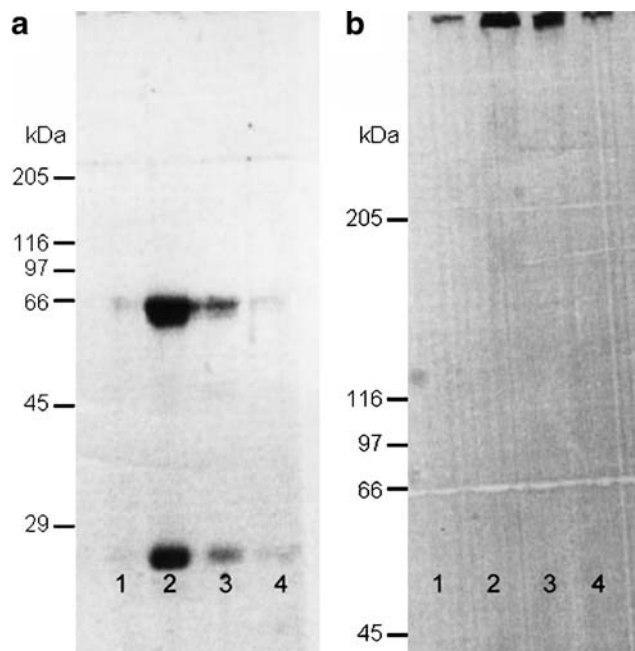


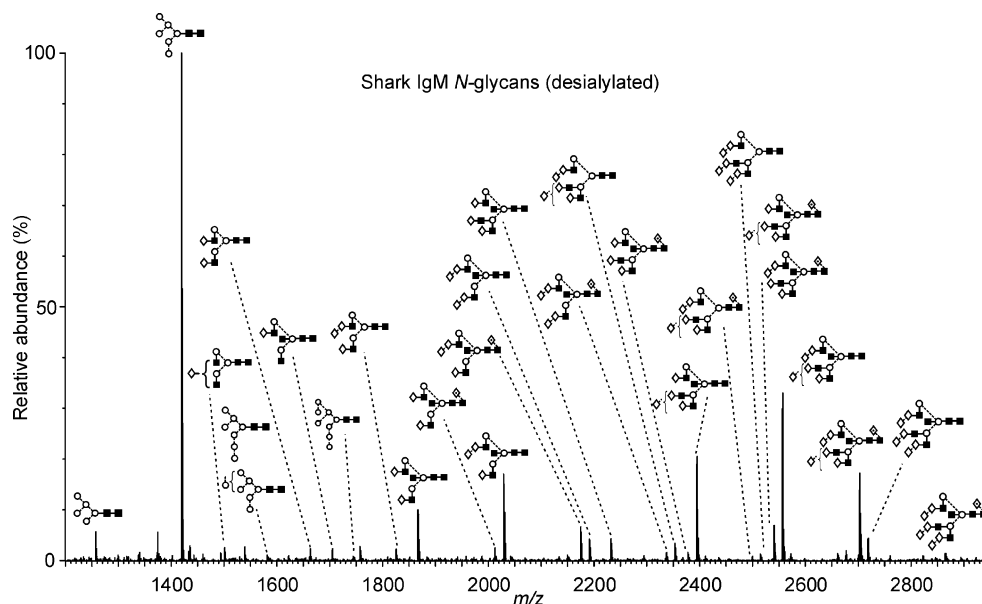
Fig 1 SDS-PAGE analysis of 19S IgM from shark serum. **A** an 8.5% acrylamide gel stained with Coomassie Blue showing reduced samples. The 70 kDa band was used for analysis of the glycans. **B** A 6% acrylamide gel showing non-reduced samples. Columns 1, 2, 3, 4 are successive samples across the peak of IgM elution from the Superose 6 column

electrospray MS profiles were virtually identical before and after treatment of the glycans with acetic acid. Compositions of the neutral glycans, as deduced from the MALDI-TOF spectra, suggested the presence of a considerable amount of α -galactose caps to the antennae and this was confirmed by incubations with β - and α -galactosidases (digests were performed in this order, because the coffee bean α -galactosidase was known to be contaminated with some β -galactosidase). Other enzyme digests with enzymes used either alone or in combination were used to determine the nature of the constituent monosaccharides. Figure 3 shows the results of incubation with *Arthrobacter ureafaciens* sialidase (ABS), ABS and bovine testis β -galactosidase (BTG) and ABS, BTG and *Streptococcus pneumoniae* β -galactosidase at a concentration that only removed β 1 \rightarrow 2-linked GlcNAc. Detailed structures of the glycans were determined by negative ion MS/MS with nanospray sample introduction into a Q-ToF mass spectrometer; they are listed in Table 1.

High-mannose glycans

The major glycan in the sample was identified as Man₆GlcNAc₂ (Structure 2, Table 1) and this was accompanied by trace amounts of Man₅GlcNAc₂ (1), Man₇-GlcNAc₂ (4) and Man₈GlcNAc₂ (7). These structures were sensitive to digestion with Jack bean α -mannosidase

Fig 2 Positive ion MALDI-TOF mass spectrum of the desialylated glycans from nurse shark IgM. Key to symbols for this and subsequent figures: *empty circle* = mannose, *filled square* = GlcNAc, *empty square* = glucose, *empty diamond* = galactose, *filled circle in an empty diamond* = fucose. Linkage positions are shown by the angle of the lines connecting the monosaccharide symbols (\mid = 1→2, \diagup = 1→3, $-$ = 1→4, \diagdown = 1→6); *full line* = β -bond, *broken line* = α -bond. Gal-GlcNAc bonds are shown as β 4- but the possibility of additional β 3-linked galactose cannot be ruled out



(data not shown). The structure of $\text{Man}_6\text{GlcNAc}_2$ (Fig. 4a) with three mannose residues in the 6-antenna and two in the 3-antenna was confirmed by the presence of the ions containing the 6-antenna (D (formal loss of the 3-antenna and chitobiose core, see structure in Fig. 4), $[\text{D}-18]^-$ and $^{0,3}\text{A}_3$ (Domon and Costello [16] nomenclature) at the same mass as in the spectrum of $\text{Man}_5\text{GlcNAc}_2$ [8]. A partial shift of these ions in the spectrum of $\text{Man}_7\text{GlcNAc}_2$ (Fig. 4b) showed that this compound was a mixture of isomers with the seventh mannose substituted on either the 3- (minor isomer) or 6-antenna. The relatively high abundance of the fragment ion at m/z 485 (D' fragment (D-type cleavage of the 6-antenna)) showed that most of the

seventh mannose was substituted in the 6-branch of the 6-antenna [8].

Complex glycans

Bi- and tri-antennary complex glycans were identified as the other type of *N*-glycan present in the mixture. The majority contained a bisecting GlcNAc residue (β 1→4-linked to the core branching mannose) and antennae terminating in α -galactose residues. The latter feature was confirmed by incubation with coffee bean α -galactosidase. The multiplicity of ion peaks in the spectrum shown in Fig. 2 was due to differing numbers of α -galactose residues

Fig 3 Positive ion MALDI-TOF mass spectrum of **a** the desialylated (ABS) glycans from nurse shark IgM; **b** the glycan profile following digestion with *Arthrobacter ureafaciens* sialidase (ABS) and bovine testis β -galactosidase (BTG); **c** the glycan profile following incubation with ABS, BTG and *Streptococcus pneumoniae* β -*N*-acetylhexosaminidase at (0.01 U/mL)

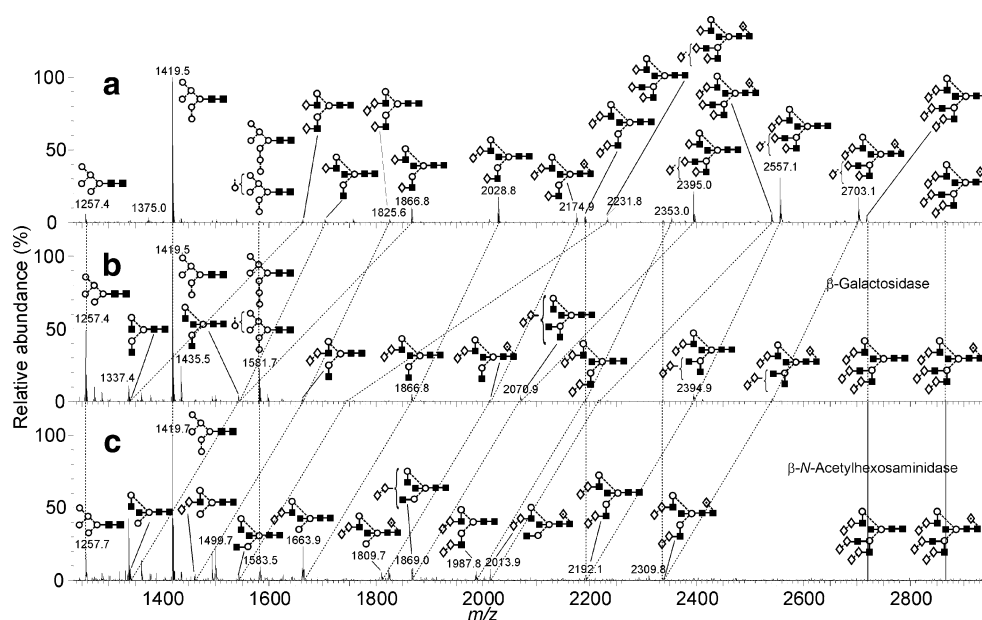


Table 1 Masses and proposed structures, shark IgM N-glycans

No.	MALDI, <i>m/z</i> ([M+Na] ⁺)		ESI, <i>m/z</i> ([M+H ₂ PO ₄] ^b)		Composition				Structure
	Found	Calc.	Found	Calc.	Hex	HexNAc	dHex	Neu5Ac	
1	1257.4	1257.4	1331.4	1331.4	5	2	0	0	
2	1419.4	1419.5	1493.4	1493.5	6	2	0	0	
3	1501.6	1501.5	1575.5	1575.5	4	4	0	0	
4	1581.5	1581.5	1655.5	1655.5	7	2	0	0	
5	1663.6	1663.6	1737.6	1737.6	5	4	0	0	
6	1704.6	1704.6	1778.6	1778.6	4	5	0	0	
7	1817.6	1817.6	1743.3	1743.6	8	2	0	0	
8	1825.6	1825.6	1899.6	1899.6	6	4	0	0	
9	1866.6	1866.7	1940.6	1940.6	5	5	0	0	
10	2012.7	2012.7	2086.7	2086.7	5	5	1	0	
11	2028.7	2028.7	2102.7	2102.7	6	5	0	0	
12	-	2179.7	2133.7	2133.7	5	5	0	1	
13	2174.7	2174.8	2248.7	2248.8	6	5	1	0	

Table 1 (continued)

14	2190.8	2190.8	2264.8	2264.7	7	5	0	0	
15	-	2341.8	2295.8	2295.8	6	5	0	1	
16	2231.7	2231.8	2305.8	2305.8	6	6	0	0	
17	2336.8	2336.8	2410.8	2410.8	7	5	1	0	
18	2352.8	2352.8	2426.8	2426.8	8	5	0	0	
19	-	2487.8	2441.9	2441.9	6	5	1	1	
20	2377.8	2377.9	2451.8	2451.8	6	6	1	0	
21	2393.9	2393.8	2467.8	2467.8	7	6	0	0	
22	-	2544.9	2498.9	2498.9	6	6	0	1	
23	2498.9	2498.9	2572.9	2572.9	8	5	1	0	
24	2514.8	2514.9	2588.9	2588.9	9	5	0	0	
25	2540.0	2539.9	2613.9	2613.9	7	6	1	0	
26	2555.9	2555.9	2629.9	2629.9	8	6	0	0	
27	-	2706.9	2660.9	2660.9	7	6	0	1	

Table 1 (continued)

28	2701.9	2702.0	2775.9	2775.9	8	6	1	0	
29	2717.9	2718.0	2791.9	2791.9	9	6	0	0	
30	-	2843.0	2807.0	2807.0	7	6	1	1	
31	2869.0	2869.0	2823.0	2823.0	8	6	0	1	
32	2822.6	2823.0	-	2897.0	10	5	1	0	-
33	2863.9	2864.0	2938.0	2938.0	9	6	1	0	
34	-	3015.0	2969.1	2969.1	8	6	1	1	

a) MS/MS spectrum not obtained; structure assigned by analogy with features present in other compounds

b) Masses of sialylated glycans (Compounds 12, 15, 19, 22, 27, 30, 31, and 34) are of $[M-H]^-$ ions

on the antennae with the result that many of the MS/MS spectra were of mixtures of isomers.

The presence of bisecting GlcNAc was confirmed by the spectrum of the glycan of composition Hex₅HexNAc₅ (**9**, phosphate adduct, m/z 1940, Fig. 5a) that was virtually identical to that of an authentic standard. The presence of the bisecting GlcNAc residue and the composition of the 6-antenna as Gal-GlcNAc was revealed by the abundant ion at m/z 670 that was produced by loss of the bisecting

GlcNAc residue from an ion, named Ion D, that contained the 6-antenna and branching mannose residue ($[D-221]^+$). Ion D itself was absent. This fragmentation pattern is diagnostic for the presence of a bisecting GlcNAc residue [9]. The most abundant ion in the spectrum, m/z 424 ($^{1,3}A_3$, Ion F), confirmed the composition of the antennae as Hex-HexNAc. An ion of low abundance at m/z 586.2, (m/z 424 + 162) that was absent from the spectrum of the reference compound corresponded to an antenna with an extra hexose (α -

Fig 4 Negative ion nanospray-MS/MS spectra of **a** Man₆GlcNAc₂ and **b** Man₇GlcNAc₂ from nurse shark, IgM. Lines connecting ions in the two spectra show the shifts specifying branching of the 6-antenna and show that two isomers of Man₇GlcNAc₂ are present

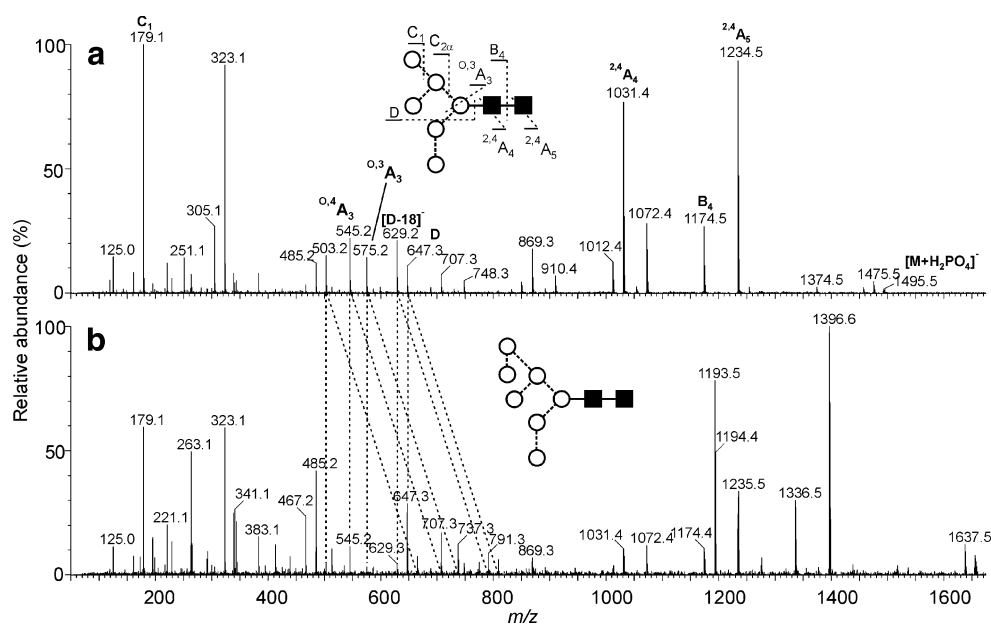
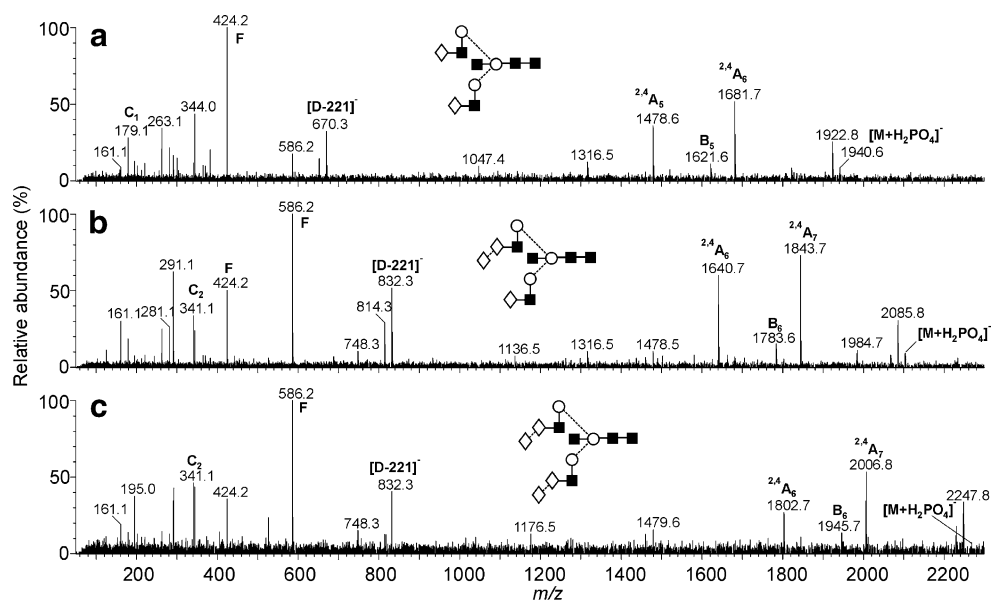


Fig 5 Negative ion nanospray-MS/MS spectra of bisected biantennary *N*-glycans with **a** zero, **b** one and **c** two α -galactose residues from nurse shark, IgM



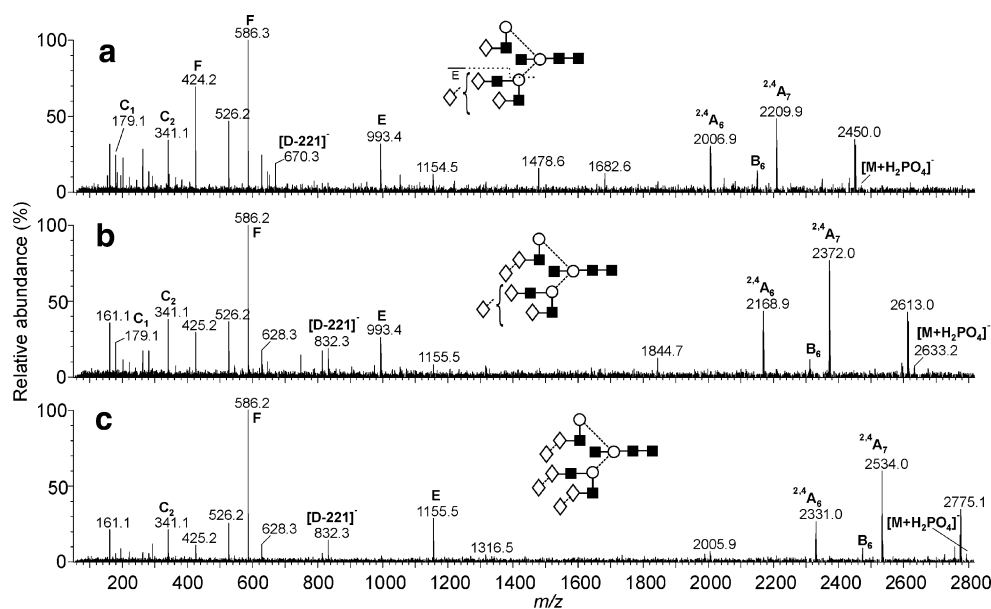
galactose) suggesting that an isomeric compound was present that had one antenna terminating in α -Gal- β -Gal while the other lacked its β -galactose residue.

The spectrum of the bisected biantennary glycan containing an additional α -galactose residue (**11**, Fig. 5b) contained a very abundant $^{1,3}A_4$ (F-type) ion at m/z 586 (α -Gal- β -Gal-GlcNAc+C₂H₄O₂) and a C₂ ion (α -Gal- β -Gal) at m/z 341 confirming this moiety and a prominent ion at m/z 832 ([D-221]⁻, m/z 670 + 162) confirming the presence of the bisecting GlcNAc and showing that the α -galactose residue was present predominantly on the 6-antenna. The compound with both antennae capped with α -galactose produced the ion at m/z 2264 (**14**) and its spectrum (Fig. 5c) contained the [D-221]⁻ ion at m/z 832 and ion F at m/z 586,

as above. No significant ions were present to suggest further extensions of the antennae.

These biantennary compounds were also present with a deoxy-hexose, presumably fucose as found in other fish species [3–5,17,18], residue attached to the 6-position of the reducing-terminal GlcNAc (Compounds **10**, **13** and **17**). However, insufficient material was available for the deoxy-hexose to be confirmed as fucose by exoglycosidase digestion. Its location was revealed by its loss during the formation of the ion that had lost carbon atoms 1, 2, 5 and 6 from the reducing-terminal GlcNAc residue, together with their substituents [9] ($^{2,4}A_6$ and $^{2,4}A_7$ ions from the compounds without and with an α -galactose residue respectively).

Fig 6 Negative ion nanospray-MS/MS spectra of bisected triantennary *N*-glycans with **a** one, **b** two and **c** three α -galactose residues from nurse shark, IgM



The most definitive spectrum of glycans from the triantennary series produced a $[M+H_2PO_4]^-$ ion at m/z 2791 (Hex₉HexNAc₆, Compound **29**, Fig. 6c). All of the antennae had the composition α -Gal- β -Gal-GlcNAc as shown by the single $^{1,3}A_4$ (F-type) ion at m/z 586. The very prominent ion at m/z 1155 of composition Hex₄HexNAc₂+101 (ion of type E [9], see Fig. 6) was typical of triantennary glycans with a branched 3-antenna. The composition of the 6-antenna (one Hex-HexNAc chain) and the presence of the bisecting GlcNAc residue were revealed by the absence of the D-type ion and presence of the $[D-221]^-$ ion at m/z 832 respectively. As with the biantennary glycans, triantennary glycans were also present that lacked some or all of the α -galactose residues both with and without a dHex residue at the 6-position of the core GlcNAc (determined by the mass of the $^{2,4}A$ ion from the reducing-terminal GlcNAc residue, Fig. 6b and c). Structures of all the compounds that were identified are listed in Table 1.

A small number of glycans were found in the electrospray spectrum with additional sialic acid (Compounds **12**, **15**, **19**, **22**, **27**, **30**, **31** and **34**) as shown by the example in Fig. 7. Incubation with *A. ureafaciens* sialidase removed the sialic acid in each case. In the spectrum shown in Fig. 7, the sialic acid anion produced the ion at m/z 290 and the ion at m/z 655 corresponds to Neu5Ac-Gal-GlcNAc (B_3 ion). The F-type ion at m/z 586 specified the other antenna as Gal-Gal-GlcNAc- and the $[D-221]^-$ ion at m/z 832 identified the bisecting GlcNAc and located the Gal-Gal-GlcNAc-antenna to the 6-position. Other sialylated glycans were identified similarly and are listed in Table 1.

Discussion

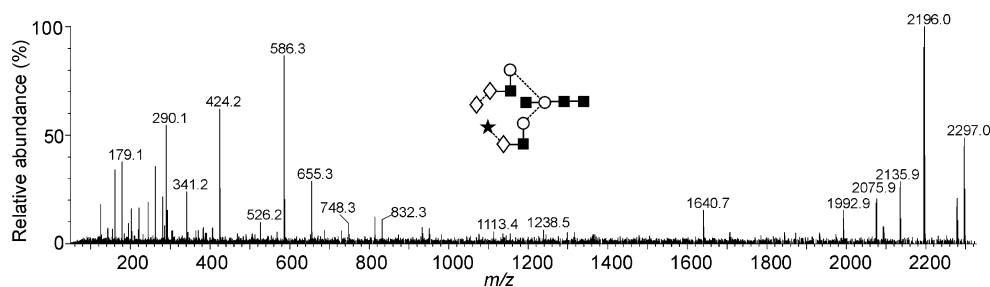
These results show that the nurse shark possesses *N*-glycans with structures very similar to those from most other vertebrate species examined. The major compounds were high-mannose structures and both bi- and tri-antennary complex glycans. Most of the complex glycans also contained a bisecting GlcNAc residue, a feature that appears not to be uncommon in *N*-glycans from trout [18], but not reflected in the glycans from IgM of the

Atlantic cod [5]. Most of the glycans had at least one antenna that terminated in an α -linked galactose residue and several of the glycans were fully substituted in this way. Fucosylation was low but, where this did occur, it was mainly on the 6-position of the reducing terminal GlcNAc residue. A few of the glycans were also found with additional sialic acid but its linkage was not determined.

The complement system in blood plasma is a major effector system in resistance to microbial infection. Complement proteins recognise and bind to microorganisms and opsonise them, so that they are destroyed by phagocytic cells. Binding of antibodies (immunoglobulins) to the microorganisms increases the activation of complement. The nurse shark has a relatively well-characterised complement system, and it would be expected that binding of IgM (antibody) to an antigen would activate the complement system *via* binding of the complement protein C1q, as occurs in mammals [19]. The binding of human C1q to immunoglobulins is well characterised, and occurs *via* recognition of clusters of charged amino acid side chains on the immunoglobulin [20]. Mammalian C1q is not regarded as a glycan-binding protein. However, a C1q orthologue has been identified in jawless fish, (lamprey), which acts as a lectin, binding to GlcNAc [21]. Jawless fish do not have immunoglobulins, but at the next evolutionary stage, cartilaginous fish (nurse shark) have both C1q and immunoglobulins. It is not known whether shark C1q recognises immunoglobulins, and if so, whether the recognition depends on protein-protein interaction, as in mammals, or whether the shark C1q acts like the lamprey C1q, as a lectin.

Another complement system recognition protein, which is well-characterised in mammals is mannose-binding lectin (MBL), which activates the complement system after binding to terminal mannose arrays on microbial surfaces. MBL can also bind to some immunoglobulins (in mouse, human) *via* terminal GlcNAc or mannose on the immunoglobulin glycans [22]. MBL has not yet been identified in nurse shark, but it is likely to be present, as it occurs in lamprey and in invertebrates [23]. Since, as shown above, the shark IgM has glycans with terminal mannose, it may activate shark complement *via* binding of C1q (if shark C1q resembles that of the lamprey) or by binding MBL

Fig 7 Negative ion MS/MS spectrum of a sialylated *N*-glycan from nurse shark Ig



orthologues. The work reported here therefore facilitates investigation of a key stage of the evolution of the immune system.

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