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Mass spectrometric strategies: providing structural clues for helminth glycoproteins

Stuart M. Haslam, Howard R. Morris and Anne Dell

Here we review current knowledge of helminth glycans and introduce parasitologists to the power of the mass spectrometric techniques that have been largely responsible for defining their carbohydrate moieties. A brief overview of glycosylation in other eukaryotes is presented, with a focus on mammalian glycosylation, to facilitate understanding of how parasite structures might be recognized as 'self' or 'foreign' by the immune system of the host.

Stuart M. Haslam
Howard R. Morris
Anne Dell*
Dept of Biochemistry, Imperial College of Science, Technology and Medicine, London, UK SW7 2AY.
*e-mail: a.dell@ic.ac.uk

Glycoproteins are abundant constituents of the outer surfaces and the excreted–secreted antigens of helminth parasites. Their structurally complex carbohydrate chains are prime candidates for host–parasite recognition events at all stages of parasitism. Indeed, glycoconjugates are increasingly being implicated in immune responses to parasite infections. In terms of their glycobiology, probably the most studied group of parasites is the schistosomes. For example, glycans carrying the Lewis^x epitope have been shown to contribute to the known T helper type 1 (Th1) cell downregulation associated with schistosomiasis in both mice[1] and humans[2], in the induction of host cytotoxic antibodies that lead to the lysis of host cells[3–5] and to the induction of hepatic granuloma[6,7]. However, little is known about the molecular basis of carbohydrate-mediated host–parasite interactions. This is largely because the structures of relatively few helminth carbohydrates have been rigorously defined and even fewer are available in quantities that permit immunological functional investigations. High-sensitivity mass spectrometry (MS) provides a means of directly addressing the first of these issues and, indirectly, the second; by amassing an increasing number of structurally defined parasite glycans we can compare structural relationships to host molecules and start to predict their potential functionality.

N- and *O*-glycosylation

There are two main types of protein glycosylation: *N*-glycosylation, in which the glycan is attached to an Asn residue present in the tripeptide consensus sequon Asn-X-Ser/Thr (where X can be any amino acid except Pro), and *O*-glycosylation, in which the glycan is attached to a Ser or Thr residue (reviewed in Refs 8,9). Glycoproteins can contain just *N*- or *O*-glycans, or a combination of both. *N*-glycans are synthesized from a common precursor (Fig. 1), which is processed by stepwise trimming and stepwise addition of new sugar residues. Trimming by α-glucosidases and α-mannosidases without any subsequent glycosyl addition to the periphery results in glycans having the composition $Man_{5\text{-}9}GlcNAc_2$. Glycans of composition $Man_{5\text{-}9}GlcNAc_2$ are designated 'high mannose' or 'oligomannose'. In invertebrates, it is not uncommon for additional α-mannose residues to be removed, giving glycans

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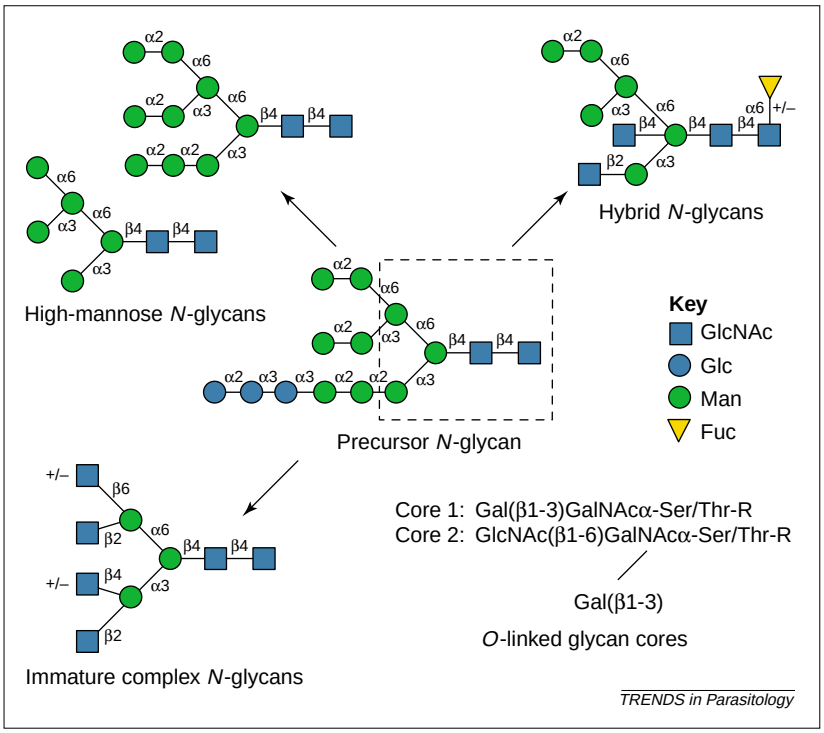


Fig. 1. Protein glycosylation. The figure illustrates how the three classes of *N*-glycans – high-mannose, hybrid and complex glycans – are derived from a common biosynthetic precursor. The trimannosyl-chitobiose core common to all *N*-glycans is outlined on the precursor *N*-glycan. The figure also includes the structure of the two most common *O*-glycan cores.

of composition $Man_{1-4}GlcNAc_2$. These small glycans are frequently referred to as 'truncated'.

Trimming by α-mannosidases together with glycosyl addition to the distal side of the core results in the formation of the most abundant class of mammalian glycans, the so-called 'complex-type' structures. These are characterized by the presence of variable numbers of antennae (usually two to four), the biosynthesis of which is initiated in the medial Golgi by the addition of GlcNAc 'stubs' to the two α-mannose residues of the core (Fig. 1). The β-mannose is also a possible site for GlcNAc attachment, and a GlcNAc residue attached to the 4-position of this mannose is referred to as a 'bisecting' residue. In plants and some invertebrates (but not mammals) the β-mannose can be substituted at the 2-position with xylose. The core can also be modified by fucosylation of the proximal GlcNAc. In mammals, this core fucose is linked to the 6-position of the GlcNAc whereas in plants it occurs at the 3-position. Invertebrates are capable of fucosylating the core at either position.

Processing in the *trans*-Golgi converts the small pool of 'core plus stubs' into an extensive array of mature oligosaccharides. In mammals, the antennae stubs (excluding the bisecting GlcNAc) are usually elongated by the addition of β-Gal to give Galβ1-4GlcNAc or 'lacNAc'. Antennae can be lengthened by the sequential addition of GlcNAc and Gal residues, resulting in tandem repeats of lacNAc (i.e. polylactosamine structures). In a restricted number of mammalian glycoproteins, β-GalNAc is added to the GlcNAc stubs in place of β-Gal and thus they have GalNAcβ1-4GlcNAc or 'lacdiNAc' antennae. Both types of antennae are common in invertebrates.

Biosynthesis of complex-type structures is completed by a variety of 'capping' reactions, the most important in mammals being sialylation and fucosylation. Capping sugars are usually α-linked, unlike the backbone residues, which are normally β-linked. Other common capping moieties include Gal, GalNAc and sulfate. Examples of capped antennae are given in Fig. 2. With the exception of sialic acid, all of the above capping moieties are also found in invertebrates.

A fourth family of *N*-glycans referred to as 'hybrid' glycans share structural features of the high-mannose and complex-type families. They retain two or more mannose residues on the six-arm of the trimannosyl core, whereas complex-type antennae are elaborated on the three-arm.

Mammalian *O*-glycan biosynthesis is initiated in the *cis*-Golgi by the addition of a single GalNAc residue to Ser or Thr. Stepwise addition of further sugar residues leads to a variety of core structures, the two most common being designated core types 1 and 2 (Fig. 1). These can be capped directly with, for example, sialic acid, or elongated with antennae that are often identical to the antennae found in complex-type *N*-glycans (Fig. 2). A detailed description of all the parasite glycan structures elucidated to date is beyond the scope of this article; however, the interested reader is directed to the excellent review of Cummings *et al.*[10], detailing schistosome glycoconjugates, and the review of Dell *et al.*[11], detailing nematode glycoconjugates.

Mass spectrometry

In the glycobiology field, no structural technique can match MS for the range of structural problems that can be addressed, the complexity of samples that can be analysed successfully and the quantity of structural information that can be obtained from subnanomolar amounts of material. The basic principles of MS applied to glycobiology are shown in Fig. 3. In the MS experiment, samples are ionized by one of several techniques (Box 1) – the most important of which are fast atom bombardment (FAB), electrospray (ES) and matrix-assisted laser desorption ionization (MALDI) – to yield molecular ions whose masses are indicative of sugar compositions. Each of these MS technologies has its individual strengths and weaknesses, and a productive structural glycobiology laboratory requires all three if the potential of MS is to be fully realized. Although compositional information can often be used to predict sequences, based on prior knowledge of biosynthetic pathways, *de novo* sequencing by MS requires fragment ions to be present in the mass spectra. These are produced if sufficient internal energy for fission of labile bonds is imparted to the molecular ions either during ionization (most commonly in the FAB-MS experiment) or via collisions with an inert gas after the molecular ions leave the ionization chamber. The latter approach is known as

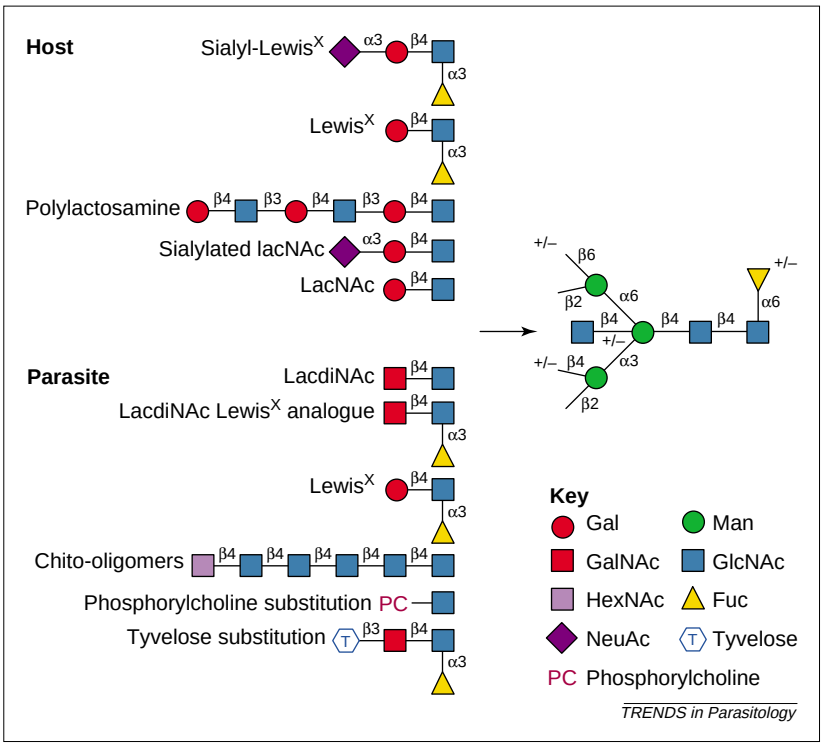


Fig. 2. Examples of *N*-glycan antennae showing capping sugars commonly found in both mammalian host and parasite glycoproteins. Note some capping groups such as Lewis^X are common to both host and parasite.

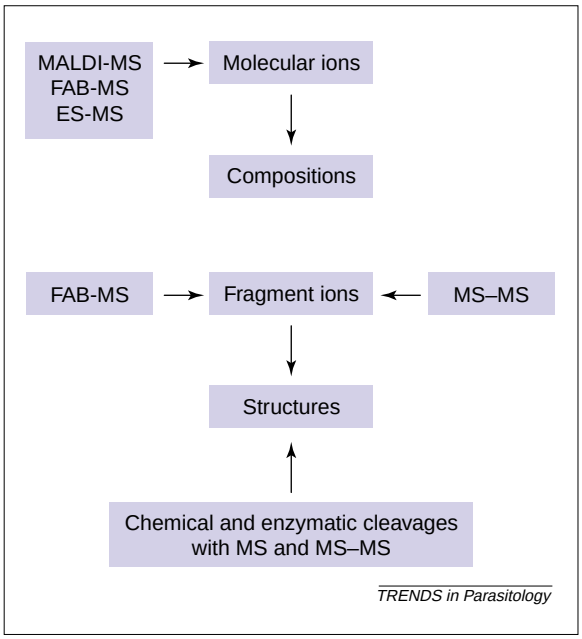


Fig. 3. Summary of an overall experimental strategy used to characterize glycans from parasites. The figure illustrates how mass spectrometry-derived molecular and fragment ions allow the assignment of both the composition and structure of glycans. Abbreviations: ES, electrospray; FAB, fast atom bombardment; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry.

tandem MS or MS–MS because two analysers are required – the molecular ion destined for fragmentation is selected by the first analyser, whereas the second analyser is used to obtain the mass spectrum of the fragment ions formed in a collision chamber placed between the two analysers. MS and MS–MS experiments rarely provide sufficient information to define carbohydrate structures rigorously. Hence, data from MS analyses of chemical and enzymatic degradations are usually required to supplement molecular and fragment ion information (Fig. 3) to define structural features such as sugar type, branching and anomeric stereochemistry.

What has MS revealed about helminth glycosylation?

Glycosylation of the following helminth species has received considerable MS attention: *Toxocara canis* and *T. cati*[12], *Trichinella spiralis*[13–15], *Haemonchus contortus*[16,17], *Acanthocheilonema viteae*[18,19], *Onchocerca volvulus* and *O. gibsoni*[19], *Dictyocaulus viviparus*[20], and *Schistosoma mansoni* and *S. japonicum*[10,21,22]. *N*-glycans have been characterized in most species but, with the exception of *S. mansoni*, *S. japonicum* and *T. canis*, *O*-glycosylation has not been subjected to similar rigorous scrutiny. Two main conclusions can be drawn from these studies. First, many parasite glycans are identical to mammalian glycans. For example, oligomannosyl structures are ubiquitous, and complex glycans bearing Lewis^X antennae have been found in *S. mansoni*, *S. japonicum* and *D. viviparus*. Second, helminth parasites synthesize a wide range of highly unusual carbohydrate moieties, many of which appear to be species and/or stage specific.

Figure 2 contains a selection of some parasite complex *N*-glycan capping groups that have been characterized. A general feature is the common use of lacdiNAc (GalNAcβ1-4GlcNAc) as opposed to lacNAc (Galβ1-4GlcNAc) for antennae formation, which produces capping groups such as the lacdiNAc Lewis^X analogue. Although such antennae are found in mammalian glycans they are restricted to a small group of glycoproteins. More novel features include: (1) chitin-oligomer-like antennae in *A. viteae*, *O. volvulus* and *O. gibsoni*; (2) the presence of phosphorylcholine in many charged glycans (note that sialic acid, which is the main contributor to charge in mammalian glycans, appears to be absent in invertebrates); and (3) *N*-linked glycans in *T. spiralis* that have lacdiNAc antennae capped with both fucose and D-tyvelose (3,6-dideoxy-D-arabinohexose). D-Tyvelose is a sugar more typically associated with bacterial cell wall lipopolysaccharides and has not been found in other eukaryotes.

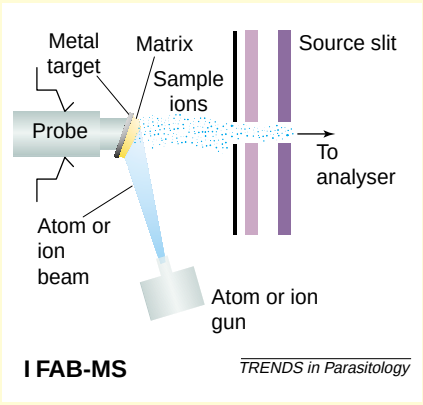
Helminth surface and excretory–secretory antigens represent the major immunogenic challenge to the host and might be the key to successful parasite defence strategies. We consider it likely that glycans of the type shown in Fig. 2 play important roles in parasitism, but this remains to be established.

Another common theme is the contribution of methyl groups to immunodominance, either as integral components of fucose and tyvelose or as substituents on other carbohydrate residues. This is exemplified by the immunodominance of

Box 1. Mass spectrometry technologies

Principles of fast atom bombardment-mass spectrometry

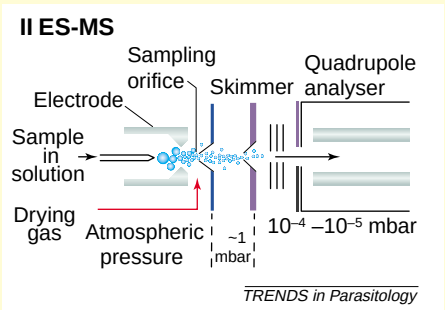
In the fast atom bombardment-mass spectrometry (FAB-MS) experiment, an accelerated beam of atoms or ions is fired from a gun towards a small metal target attached to the end of a probe (Fig. I). The target is loaded with a viscous liquid (the matrix) in which the sample to be analysed is dissolved. When the atom or ion beam collides with the matrix, sample molecules are sputtered out of the matrix into the high vacuum of the ion source. Significant numbers of these molecules are ionized during the sputtering process and, during ionization, internal energy is imparted to the molecules, resulting in fragmentation of labile bonds. A particular strength of FAB-MS is its ability to provide both compositional (via the molecular ions) and sequence information (via the fragment ions) from complex mixtures of glycans in a single experiment. Consequently, FAB-MS is a powerful tool for screening biological material such as detergent extracts of whole parasites, excretory–secretory products and affinity purified glycoproteins for their glycan content.



Screening strategies are based on the analysis of permethylated derivatives, which yield molecular ions at high sensitivity, irrespective of the type of ionization, and which, in the FAB-MS experiment, reliably afford characteristic fragment ions (A-type ions) resulting from cleavage on the reducing side of HexNAc residues. The 'map' of A-type ions generated from a mixture of glycans defines all the non-reducing sequences present in the sample[a]. Our studies on schistosomal egg glycans exemplify the power of screening strategies[b].

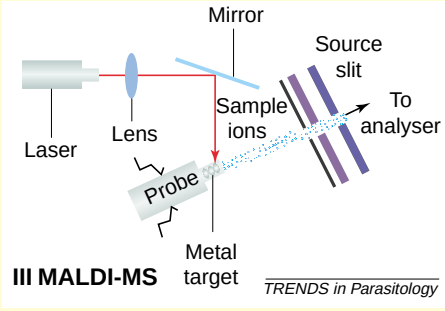
Principles of electrospray-MS

In electrospray-MS (ES-MS), a stream of liquid containing the sample of interest is introduced into the atmospheric pressure ion source of a mass spectrometer via a metal-tipped glass capillary (Fig. II). An aerosol of highly charged microdroplets is generated in the source, which then traverses a series of skimmers, encountering a drying gas, the net effect of which is the creation of gaseous ions, devoid of solvent, whose charge depends on the number of ionizable groups in the molecule. The ionization process in ES-MS is very gentle, resulting in little or no fragmentation. To overcome this problem, most ES instruments have tandem analysers to allow MS–MS experiments. The most powerful technology for ES-MS–MS is the quadrupole orthogonal acceleration time of flight (Q-TOF) mass spectrometer[c,d]. This instrument, which was conceived in our laboratory in the mid 1990s for ultra-high sensitivity low femtomole/attomole range biopolymer sequencing, has already had a major impact on proteomics research and is playing an increasingly important role in structural glycobiology[e–g].



Principles of matrix-assisted laser desorption ionization-MS (MALDI-MS)

Matrix-assisted laser desorption ionization-MS (MALDI-MS) is arguably the most sensitive of the three technologies. In MALDI the sample is embedded in a low-molecular weight, ultraviolet-absorbing matrix, and ionization is effected by a pulsed laser (Fig. III). The matrix absorbs the laser pulse and enough energy is transferred to the sample, via mechanisms that are not well understood, to enable formation of molecular ions. The MALDI experiment yields few fragment ions and, unlike ES-MS, MALDI technology does not readily permit MS–MS experiments. Hence, MALDI-MS is the pre-eminent technique for screening for molecular ions, especially when high throughput and the highest sensitivity is demanded.



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trifucosylated *N*-glycan cores in *H. contortus*[16], the oligofucosyl appendages on schistosome core type 1 and 2 *O*-glycans[21], the lacdiNAc antennae capped with both fucose and D-tyvelose in *T. spiralis*[13] and the presence of methyl-substituted fucose and galactose in *Toxocara O*-glycans[12].

Concluding remarks

Advances in biopolymer MS in the past 20 years have been truly staggering. As described above, FAB-MS, MALDI-MS and ES-MS are powerful techniques for glycopolymer structure analysis. Each has its own strengths. For example, MALDI-MS would be the method of choice for ultra-high-sensitivity molecular weight profiling, FAB-MS is ideally suited to screening for non-reducing epitopes in biological samples, and ES-MS–MS on the quadrupole orthogonal acceleration time of flight (Q-TOF) provides the most sensitive means of sequencing peptides and glycopeptides. With an appropriate choice of experimental strategy, many structural problems can be addressed successfully with any one of the three ionization methods. Thus, for many researchers, access to particular instrumentation and expertise might be more important than factors such as relative sensitivities or whether collisional activation is required to generate fragment ions. The structures shown in Fig. 2 almost certainly represent only the first of many novel helminth glycans. Thus, MS strategies will continue to provide the vital structural underpinning of research aimed at understanding host–parasite interactions.

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