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Glycomics Profiling of Heparan Sulfate Structure and Activity

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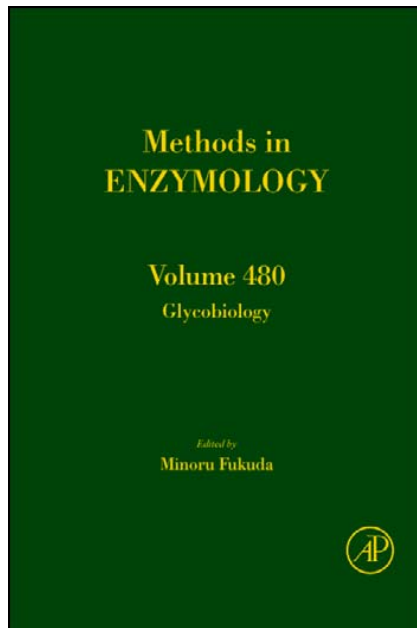
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GLYCOMICS PROFILING OF HEPARAN SULFATE STRUCTURE AND ACTIVITY

Jeremy E. Turnbull, Rebecca L. Miller, Yassir Ahmed,
Tania M. Puvirajesinghe, *and* Scott E. Guimond

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Abstract

The heparan sulfate (HS) family of glycosaminoglycans are highly complex and structurally diverse polysaccharides with information encoded within the chains that imparts the ability to bind selectively to a wide range of proteins—the “HS interactome”—and to regulate their biological activities. However, there are two key questions which need to be addressed; first, the extent of structural variation of expressed HS structures—the “heparanome”—in specific biological contexts and second, the degree of functional selectivity exerted by these structures in regulating biological processes. There is a clear need to develop more systematic and high throughput approaches in order to address these questions. Here, we describe a cohort of protocols for profiling different aspects of HS structure and activity, focusing particularly on disaccharide building blocks and larger oligosaccharide domains, the latter representing the functional units of HS chains. A range of other complementary methods in the literature are also discussed. Together these provide a new and more

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comprehensive toolkit to investigate HS structure and activity in a higher throughput manner in selected biological systems. The implementation of such a glycomics strategy will enable development of a systems biology view of HS structure–function relationships and help to resolve the significant puzzle of the extensive interactome of HS, which remains a key question in the glycobiology field. We anticipate that the next decade will see major advances in our understanding of the complex biology of HS.

1. OVERVIEW

1.1. HS structure and biosynthesis

Heparan sulfate (HS) is a member of the glycosaminoglycan (GAG) family of linear polysaccharides made up of repeating disaccharide unit backbones onto which are superimposed specific modification patterns, most notably addition of sulfate groups. Within this family, HS has the most highly varied structure, with polymorphic sulfated sequences expressed found in its chains which are responsible for the many protein binding and regulatory properties of HS (Turnbull *et al.*, 2001). HS chains are normally attached to core proteins in the form of HS proteoglycans (HSPGs), which are located at the cell surface and in the extracellular matrix. HS biosynthesis involves a complex set of Golgi enzymes which initially produce a nonsulfated polysaccharide chain precursor, heparosan. A sequential series of modifications then follows which superimpose complex patterns of sulfate group addition and uronate epimerization at selective positions. Most important, the system is not template-driven and the reactions only proceed to a partial extent, resulting in a high degree of structural diversity of HS sugar sequences. There is an ordered structure to HS chains in which domains with differing types and density of modifications are spaced apart along the molecule (Turnbull and Gallagher, 1991). Domains where the heparosan has undergone relatively few modifications consist mainly of GlcA-GlcNAc repeats, and are called *N*-acetylated (NA) domains. These act to space apart the highly modified, sulfated domains (S-domains; typically 3–8 disaccharide units in length) in which extensive modifications occur, especially *N*- and *O*-sulfate group additions and epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA). Flanking domains that have alternating *N*-acetylated and *N*-sulfated disaccharides (NA/NS domains) are also present (Lindahl *et al.*, 1998; Turnbull *et al.*, 2001).

Variant HS chain modifications provide structural diversity that underpins its many protein binding and regulatory properties. The potential variations in even short saccharide sequences are very high, making HS a very information dense molecule (Nugent, 2000), though the theoretical

variations are restricted by biosynthetic constraints. Reproducible differences in HS structure have been observed at both the tissue (Brickman *et al.*, 1998; Ford-Perriss *et al.*, 2002; Maccarana *et al.*, 1996) and cell level (Kato *et al.*, 1994; Sanderson *et al.*, 1994), and upon cell transformation (Jayson *et al.*, 1998). Identical core proteins can display different HS structures when expressed by different cell types (Kato *et al.*, 1994; Sanderson *et al.*, 1994). Furthermore, it is increasingly evident that HS structures are biosynthesized in a dynamic manner: HSPGs have a rapid turnover (half-life 3–4 h) in cultured cells (Yanagishita and Hascall, 1984); cells can alter the HS structures they make, for example, in response to extracellular signals such as growth factors (Schmidt *et al.*, 1995); and changes in HS structure have been shown to occur over short time spans in developing tissues (Ford-Perriss *et al.*, 2002). HS is thus not as a single molecule but is in fact a highly diverse family of related molecules with dynamic and specific functional roles (Turnbull *et al.*, 2001).

1.2. Heparan sulfates: A family of multifunctional cell regulators

The HS family is now viewed as multifunctional players with vital roles in cell regulation (Turnbull *et al.*, 2001). Their strategic location at the cell surface and in the extracellular matrix, positions them ideally for selective regulatory interactions with many proteins (Ori *et al.* 2008; Powell *et al.*, 2004). These interactions can result in functional regulation of protein activities and are critical for a range of physiological processes (Bishop *et al.*, 2007). Genetic studies in model organisms have clearly demonstrated that HS is essential for many aspects of development and normal physiological functions (Lander and Selleck, 2000).

HS interacts with an extraordinary range of proteins (the HS “interactome”) including growth factors, enzymes, extracellular matrix proteins, and proteins found on the surface of pathogens (Ori *et al.* 2008). In many examples of HS–protein interactions there is evidence that these interactions are selective to varying degrees and serve regulatory roles. A principal role of HS is to act as a “catalyst of molecular encounters” (Lander *et al.*, 1999). The mechanisms of action of HS are an active area of investigation which has revealed a number of distinct modes of action in mediating protein–protein interactions, including altering protein conformations (e.g., antithrombin III), increasing protein stability and restricting protein mobility, acting as a cell surface receptor, and acting as a template for controlled assembly of protein complexes (Turnbull *et al.*, 2001). Probably one of the best studied examples of the latter is the action of HS as a coreceptor for the fibroblast growth factor (FGF) family, where it modulates

the interaction of FGF with its receptor tyrosine kinase (Ornitz *et al.*, 1996; Rapraeger *et al.*, 1991; Yayon *et al.*, 1991). HS activates signaling by participating in the formation of a ternary complex, mediated by specific interactions of the HS with both the growth factor and its receptor, and the presence or absence of specific sulfate groups within HS can dictate which members of the FGF family can signal through which receptors (Guimond and Turnbull, 1999; Pye *et al.*, 1998).

1.3. Decoding HS structure–function: Toward glycomics approaches

Two key questions in the field are now the extent of natural diversity of HS structures—the “heparanome”—in specific biological systems (Turnbull *et al.*, 2001) and the level of sequence specificity or selectivity in terms of regulation of biological responses (Lindahl and Ping, 2009). It has become increasingly clear that HS does not influence protein functions in a “digital” on/off manner via highly specific single sequences with single functions, but rather via “analog tuning” in which ensembles of restricted motifs are able to exert a range of modulatory functions. Further work is now clearly required to decode HS structural diversity and how this relates to functional properties in a systematic manner. Glycomics technologies have recently been expanding in the wider glycobiology field (Turnbull and Field, 2007), and strategies appropriate for HS can now be envisaged which integrate a variety of methods in order to develop a systems biology view of the structural selectivity underlying the diverse functions of HSPGs.

The first step for this type of strategy is isolation of HS from the appropriate cells or tissues under study. Second, the “building blocks” present can be determined by quantitative disaccharide compositional analysis, permitting a first (basic) level of comparison of tissue and cell type specificity of HS structures. This is typically achieved using HPLC methods employing UV or fluorescence detection, or by mass spectrometry, using authentic standards. Third, and increasingly important, the higher order organization of disaccharide building blocks into oligosaccharide level domains can be profiled (or “mapped”) using a variety of methods including gel electrophoresis, gel filtration, HPLC, and mass spectroscopy. Finally, it is also possible to profile the bioactivities of particular saccharides, allowing screening and determination of the functional specificity of particular saccharides. Here, we present protocols for a number of methods in this type of work flow and further information on additional alternative methods.



2. EXPERIMENTAL

2.1. Rapid purification of HS

In order to study the variant spatial and temporal regulation of HS biosynthesis in cells and tissues, efficient methods for extraction of HS are required to facilitate subsequent structural analysis. Isolation of HS and other GAGs from tissues can be achieved through several means. The most common involves the dissolution of tissues using proteases, detergents, and/or chaotropic salts. Alkaline treatment of tissues can also be used to release GAG chains from their protein cores. The exact method used is chosen based on the amount and nature of the starting material and the availability of equipment and reagents. These methods for the isolation of HS are more suited to use on larger amounts of tissues or have multiple, lengthy steps. Previous methods could also suffer loss of material during transfer steps. Use of the recently developed RIP (*Rapid Isolation of Proteoglycans*) method to isolate HS from small cell or tissue samples overcomes these problems (Guimond *et al.*, 2009).

2.1.1. RIP method for isolation of proteoglycans

1. Tissues are ground in a tissue homogenizer with 1 ml of Trizol reagent for every 100 mg of wet weight of starting tissue. The samples are left for 5 min at room temperature (RT), and then 0.2 ml of chloroform is added per 1 ml of Trizol originally used. Samples are shaken vigorously for 15 s and then left for 3 min at RT. Centrifuge samples at no more than $12,000 \times g$ for 15 min at 4 °C.
2. Remove the upper, aqueous layer containing the proteoglycans as well as RNA. Dilute 1:10 in water and apply to DEAE-Sephacel (100 μ l beads/100 mg starting material; GE Life Sciences) or a comparable weak anion exchange matrix. This can be done as a batch absorption or as a column. Wash with 10 column volumes of phosphate buffered saline (PBS) followed by 10 column volumes of PBS (0.25 M NaCl) and elution with 10 column volumes of PBS (2 M NaCl). Desalt samples and freeze dry. Samples can be stored at -20 °C.
3. After the first weak anion exchange step, the samples should be reconstituted in 100 μ l of HPLC-grade water and passed through a 5000 MWCO spin filter to remove any impurities that were not removed in the previous steps that may affect subsequent enzyme and labeling steps.
4. Digestion and removal of contaminating molecules is achieved by the sequential addition of enzymes, followed by weak anion exchange chromatography and desalting. Common choices are DNase, RNase, chondroitin ABC lyase, neuraminidase, keratanase, hyaluronidase, and proteases, depending on the molecule and level of purity required.

The RIP method is much faster than previous methods, with samples ready for initial weak anion exchange chromatography in as little as 30 min and ready for many structural analyses in 2 days (Guimond *et al.*, 2009). It also requires only one transfer before the first weak anion exchange step, thus minimizing sample loss. The weak anion exchange step and subsequent enzyme steps are common to all methods of purification. This method can also be used to isolate other GAGs by altering the enzymes used in the additional purification step. It can also be used on cultured cells. The main disadvantage is the nature of the phenol/chloroform limits the method to small amounts of tissue (up to 1 g) to avoid the handling and accumulation of large amounts of chemical waste. Figure 4.1 shows an example of analysis of HS derived from mouse tissues by the RIP method.

2.2. Profiling disaccharide composition

The most common method of profiling HS from different sources is the analysis of disaccharides. This type of analysis is an essential first step for characterization of HS. Although common disaccharides are present in different tissues, the proportion of each disaccharide may vary quantitatively, resulting in structural differences between tissues. Disaccharide analysis is most commonly achieved using a mixture of heparinases I, II, and III, derived from *Flavobacterium*, which are able to cleave HS chains to disaccharide products via an elimination reaction (Hovingh and Linker, 1970; Linhardt *et al.*, 1990). This introduces a C4–C5 unsaturated double bond in the uronic acid residue at the nonreducing end, making direct absorbance measurements possible at 232 nm (molar extinction coefficient of $5500 \text{ M}^{-1} \text{ cm}^{-1}$; Linhardt *et al.*, 1988). Analysis of the resulting disaccharide structures is most commonly undertaken using HPLC separation techniques with UV detection.

However, extraction of HS from small amounts of cells or tissues often produces only small quantities of purified HS sample, making more sensitive analysis techniques essential. High sensitivity detection can be achieved by labeling the products of heparinase digestion, with newly created reducing ends, with fluorescent tags having amine or hydrazide functional groups. Fluorophores which have been coupled to HS disaccharide structures include 2-cyanoacetamide (Toyoda *et al.*, 1997), 2-aminoacridone (AMAC; Deakin and Lyon, 2008; Hitchcock *et al.*, 2008a,b; Kitagawa *et al.*, 1995), 2-aminobenzamide (Kinoshita and Sugahara, 1999; Yamada *et al.*, 1999), and 2-aminobenzoic acid (Sato *et al.*, 2005; Turnbull *et al.*, 1999; Volpi *et al.*, 2009). Reported sensitivities are in the picomol range, with enhanced sensitivity possible using laser-induced fluorescence, often coupled to capillary electrophoresis (Hitchcock *et al.*, 2008a; Karamanos *et al.*, 1996; Lamari *et al.*, 1999; Militopoulou *et al.*, 2002).

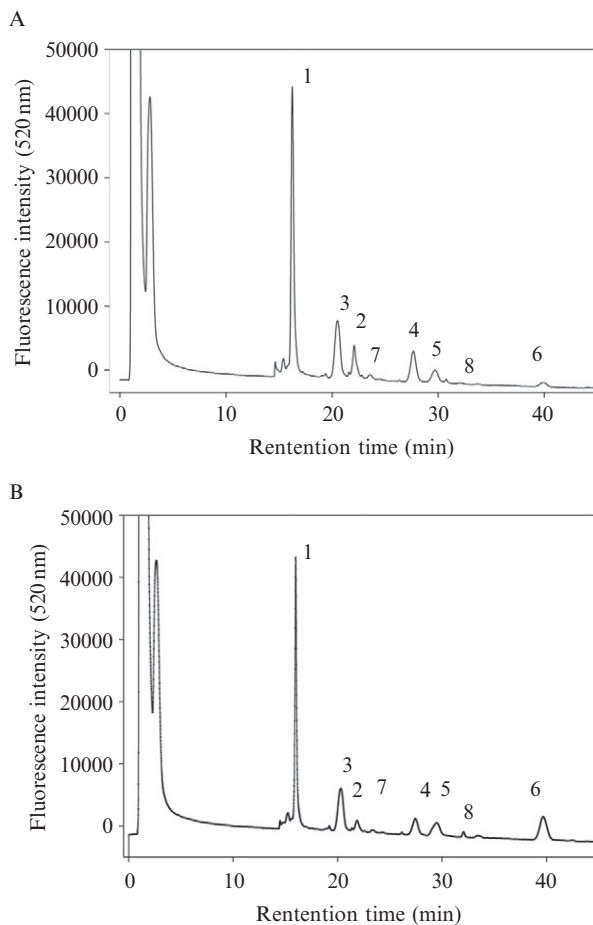


Figure 4.1 Disaccharide compositional profiling of HS from different mouse tissues using RIP extraction and fluorescence detection. HS is purified from 100 mg of each tissue by RIP, digested with heparitinases, labeled with BODIPY hydrazide, and separated by HPLC. Representative data are shown for the tissues: (A) spleen and (B) liver. Disaccharide structures are identified by comparison of retention time with authentic standards (Dextra Ltd). Disaccharide standards are (1) UA-GlcNAc, (2) UA-GlcNAc(6 S), (3) UA-GlcNS, (4) UA-GlcNS(6 S), (5) UA(2 S)-GlcNS, (6) UA(2 S)-GlcNS(6 S), (7) UA(2 S)-GlcNAc, and (8) UA(2 S)-GlcNAc(6 S).

A more recently developed fluorescent tag is 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid (BODIPY) hydrazide (Atrih *et al.*, 2009; Skidmore *et al.*, 2006, 2010). This label has several advantages including a detection limit of ~ 100 fmol for an HS disaccharide mixture, which provides a >1000 -fold increase in sensitivity over the use of UV absorbance and 10- to 100-fold increase in

sensitivity over previous fluorescent labels. The method for labeling HS disaccharides with BODIPY fluorescent tag is described below.

Another recent technology is quantification of disaccharides using off-line and online mass spectrometry. The advantages of mass spectrometry are accurate molecular mass confirming the compound, as contaminants from extracts can elute at the same position as the known standard on an HPLC system and the other reason being the sensitivity. Even though six of the disaccharides have isomeric structures, by use of collision-induced dissociation (CID), it is possible to differentiate between them. Quantification by off-line mass spectrometry on biological samples has been achieved using tandem MS, since the ratios of CID products giving a complete quantification analysis without the use of standards. Off-line disaccharide analysis takes minutes and requires no chromatography cleanup (Desaire and Leary, 1999, 2000; Saad and Leary, 2003). Online mass spectrometry has advantages over off-line due to the chromatographic separation; graphitized carbon columns and hydrophilic interaction liquid chromatography (HILIC) may not give full disaccharide separation (but can provide analysis with reference to standards), whereas reverse-phase ion pairing (RPIP) is able to separate all eight common disaccharides, though the ion pairing reagents can cause a small decrease in sensitivity and quality of the spectrum (Zhang *et al.*, 2009). LC-MS using RPIP has also been used for quantitation of disaccharides reductively labeled with aniline-containing stable isotopes (Lawrence *et al.*, 2008).

2.2.1. Fluorescence HPLC analysis of HS disaccharide composition using BODIPY label

1. Tissue and cell samples are prepared using the RIP protocol, as described above (Guimond *et al.*, 2009).
2. A preclearing step is carried out to remove low MW contaminants and improve labeling efficiency. Samples are aliquoted into Ultrafree 0.5 spin filters (NMWL 5 kDa; Millipore) and centrifuged for 5 min at 12,000 rpm.
3. HS oligosaccharides are digested into disaccharides using a mixture of heparinases I, II, and III (Ibex Ltd.). Complete digestion with heparinase enzymes is achieved by incubating at 37 °C using initial digestion with heparinase I for 2 h, followed by heparinase III for 2 h and finally addition of heparinase II, with an overnight incubation. Heparinase enzymes were used at 2.5 mU each (in 10 μ l of 5 \times heparinase buffer, 500 mM sodium acetate, 0.5 mM calcium acetate, pH 7.0). Total volume used is typically 50 μ l, and these conditions are suitable for digestion of up to 1 mg of sample.
4. For reducing-end labeling with the BODIPY dye, samples are initially lyophilized and centrifuged in a 500- μ l Eppendorf tube. For the labeling

steps, 10 μl of 5 mg/ml BODIPY hydrazide in methanol is added to the lyophilized sample. Methanol is evaporated using a vacuum centrifuge set at 36 °C for 30 min.

5. The labeling reaction is carried out in dimethyl sulfoxide (DMSO): glacial acetic acid (17:3, v/v) and incubated at RT for 4 h.
6. The imine (Schiff's base) structures are then reduced to stable derivatives by incubating with 5 μl of 1 M sodium borohydride solution (Sigma-Aldrich) for 30 min at RT.
7. Samples are then flash frozen using liquid nitrogen and lyophilized. Before separation and analysis, samples are reconstituted with DMSO: water solution (1:1, v/v) and resuspended in sample loading solution (150 mM NaOH).
8. BODIPY-labeled unsaturated HS disaccharides are separated and analyzed using a linear sodium chloride gradient over an isocratic sodium hydroxide solution mobile phase, using a Propac PA1 column (Dionex) on an HPLC system. The column is first equilibrated in sample loading solution (150 mM NaOH). The fluorescently labeled disaccharides are then loaded using 150 mM NaOH eluent, with a 10- to 20-min wash step during which the free label elutes and baseline fluorescence levels are restored. The disaccharides are then eluted using a linear gradient of 0–1 M sodium chloride (in 150 mM NaOH), over 30 min at a flow rate of 2 ml/min. Fluorescence measurements are recorded using in-line fluorescent detection at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. The column is washed using 2 M NaCl in 300 mM NaOH.

This approach has been used to rapidly profile the disaccharide composition of tissue HS and small amounts of HS from cell culture (Guimond *et al.*, 2009). An example of disaccharide compositional profiling of HS from different mouse tissues using RIP extraction, BODIPY labeling, and strong anion exchange (SAX)-HPLC with fluorescence detection is shown in Fig. 4.1. Previously calculated correction factors are applied to quantitate the observed disaccharides, since there are differences in efficiency of labeling (Skidmore *et al.*, 2006).

2.3. Profiling HS oligosaccharides

Following disaccharide compositional analysis, the next stage is to examine the structural profiles of larger oligosaccharides obtained by partial cleavage of HS chains, in order to provide insights into the higher order domain structure. Previous work has exploited methods including gel electrophoresis (Linhardt *et al.*, 1988; Turnbull and Gallagher 1988), gel filtration (Turnbull and Gallagher, 1990, 1991), and HPLC methods including SAX-HPLC (Guimond and Turnbull, 1999; Powell *et al.*, 2010) and

reverse-phase HPLC (Thanawiroon and Linhardt, 2003). More recently, strategies to profile differences in oligosaccharide domains have been developed employing online mass spectrometry techniques. These strategies look at the total ion abundances from online separations, which yields information on the structures present, including number and types of monosaccharide units, the presence of unsaturated and saturated uronate residues, number of sulfates, number of acetates, and also presence of linker region structures. However, quantitative comparisons by total ion abundance are not possible, since each structure can give different intensities due to differential ionization properties, and standards are simply not available for all structures. However, comparisons of tissue oligosaccharides with the additional data from tandem MS are quantifiable, by comparison between the B-, C-, and Y- type ions based on the nomenclature of Domon and Costello (1988). This strategy has recently been used to compare differences in total ion abundance oligosaccharide patterns between normal bovine cartilage and diseased adult human cartilage, and agreed with similar findings that the sulfation of CS changes with age (Estrella *et al.*, 2007; Hitchcock *et al.*, 2008b). It has also been used to study differences between HS chains between organs (Staples *et al.*, 2009). Thus, this developing methodology is providing structural insights into the differences between HS from different tissues using microgram amounts of sample.

2.3.1. Liquid chromatography electrospray mass spectrometry

Oligosaccharide masses were analyzed using liquid chromatography electrospray mass spectrometry (LC-ESI MS) using graphitized carbon columns.

1. SAX-HPLC-purified HS saccharides were desalted and separated using a porous graphitized carbon column (100 mm × 0.32 mm, 5 μm; Thermo Hypersil, Runcorn, UK). Eluent A was 10 mM ammonium bicarbonate in HPLC-grade water, and eluent B was 80% acetonitrile/10 mM ammonium bicarbonate.
2. The samples of varying concentrations were injected and a linear gradient of 100% eluent A to 40% eluent B employed over 40 min at a flow rate of 4 μl/min.
3. Elution into the electrospray source of the mass spectrometer (LCQ-XP+ Thermo Finnigan) was at a flow rate of 4 μl/h, via a 1-μm tip heated to 270 °C.
4. The negative ion mass spectrum of the sample was recorded using a spray voltage of 3.5 kV.

Measuring the total ion count allows a profile of the separated oligosaccharides to be obtained, for example, allowing comparison of different size-fractionated pools of HS oligosaccharides (Fig. 4.2). The masses of saccharides can be examined in each peak to determine a profile of the

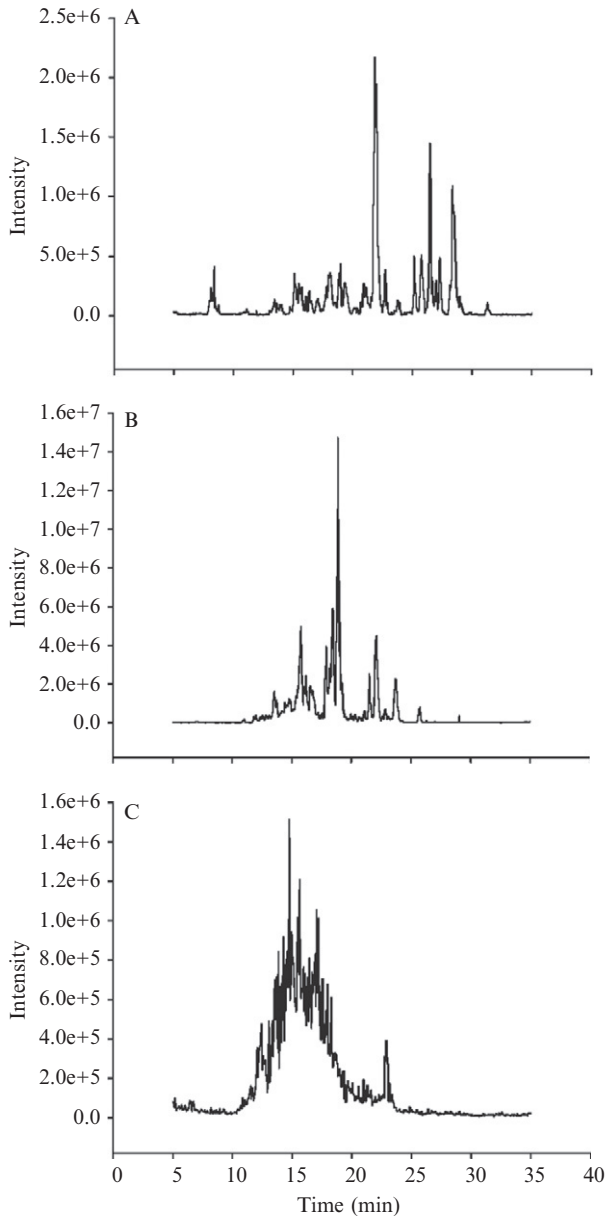


Figure 4.2 Graphitized carbon column LC-MS of size-defined gel filtration fractions from porcine mucosal HS digested with heparinase III. PMHS was digested using heparinase III and the oligosaccharides further separated using gel filtration chromatography on 30 XK16 preparation grade column (30 mm \times 200 cm, bead size 34 μ m). Fractions corresponding to particular size-defined peaks were collected, freeze dried,

oligosaccharide constituents. This type of analysis can provide accurate profiling information on the types of structures found in a particular HS chain and permits comparisons to be made between samples (Staples *et al.*, 2009).

2.3.2. Software tools for HS oligosaccharide characterization

The analysis of GAGs is most often undertaken using HPLC and mass spectrometry techniques. HPLC techniques alone provide constructive data via quantitative compositional analysis, and also comparative profiling, and the latter can be supported by mathematical methods for chemogenic comparisons (Puvirajesinghe *et al.*, 2009). More recently, a leading technique for oligosaccharide profiling, compositional analysis, and identification of rare structures is mass spectrometry. Both ESI and MALDI (Laremore *et al.*, 2007; Tissot *et al.*, 2007) have been employed, ESI providing soft ionization and LC–MS options for accurate analysis and MALDI providing rapid analysis, particularly for backbone structures. Now that mass spectrometry in this field has developed, a stream of oligosaccharide data are beginning to emerge, following strategies that proteomics have adopted. Consequently, bioinformatics programs for the analysis of mass spectrometry spectra are becoming a valuable asset for data analysis. These programs include HOST (Saad and Leary, 2005) and a modified version of Glycoworkbench adapted for GAG analysis (Tissot *et al.*, 2008), based on the Domon and Costello (1988) nomenclature. HOST is a simple Excel software program which uses a combination of clues from MS, MSMS, and enzymatic digestion. For example, heparinase I and III digestion provide clues to the oligosaccharide sequence because of their specificities, assisting MS and MSMS data collection from the same oligosaccharide products. Both data from MS and tandem MS give clues to the sequence, MS revealing the number of sulfates, acetates, and disaccharide units by the $[M - H] - 1$ ions and MSMS producing fragmentation patterns based on known comparisons (Saad and Leary, 2005). Combined with compositional analysis, HOST will give a score between 1 and 0, with 1 being the correct sequence, and 0 being an incorrect sequence. The only other data interpretation software available to date is Glycoworkbench (Ceroni *et al.*, 2008). This software analyses spectra by assigning mass ions for both MALDI and ESI data sets. Analysis of the MSMS spectra is also possible, and gives a graphical representation of all

and subjected to LC–MS analysis using a graphitized carbon column (100 mm × 0.32 mm, 5 μm; Thermo Hypersil, Runcorn, UK) and an LCQ-XP+ Thermo Finnigan instrument as described in Section 3. Each fraction (A)–(C) was run using the same conditions 0–40% acetonitrile and 10 mM ammonium bicarbonate. (A) LC–MS of a dp4 fraction, (B) LC–MS of a dp6 fraction, and (C) LC–MS of a dp10 fraction.

possible cleavage products, in which the user can pick the number of cleavage events. These data sets have all been verified using known heparin and chondroitin oligosaccharides. It can also be used independently to build structures *in silico* from building blocks, and examine their theoretical fragmentation patterns. With mass spectrometry becoming an established tool for the analysis of GAGs, it may be expected that data production will increase exponentially, with consequent requirements for rapid and automatic interpretation of data.

2.4. Profiling HS oligosaccharide function

Natural heparan sulfate (HS) oligosaccharide libraries are an important source for exploring HS structure–activity relationships (SAR) and now permit the development of functional glycomics studies. Libraries of HS oligosaccharides can be produced by partial degradation of tissue-derived HS polysaccharide chains and chromatographic fractionation of the resulting oligosaccharide mixtures (Powell *et al.*, 2010). The initial step in HS oligosaccharides production is partial digestion, for example, with heparinase III (to isolate predominantly the sulfated domains), followed by fractionation based on hydrodynamic volume, using size exclusion chromatography (SEC). Subsequent to SEC fractionation, selected size-defined oligosaccharide pools can be subjected to SAX-HPLC, and the resulting products desalted and quantified. These HS oligosaccharide fractions have been shown to contain purified or partially purified oligosaccharides. A brief protocol is provided below, and a comprehensive protocol has been described by Powell *et al.* (2010).

2.4.1. Method for HS oligosaccharide library production

1. Partial enzyme digestion is performed on a purified HS sample after resuspension in heparinase buffer [100 mM sodium acetate, 0.1 mM calcium acetate (pH 7.0)]. Trial digestion is conducted first in order to determine a suitable amount of heparinase III to use and the time required for digestion to the required extent. Bulk digestion to completion of 400 mg HS was conducted by heparinase III at 0.1 mU/mg (5 mU/ml) for 2 h, then 10 mU heparinase III was added and incubated for at 37 °C for 22 h, and then further 10 mU was added for 2 h. The reaction was stopped by boiling for 2 min; partially digested saccharides were lyophilized and stored at –20 °C.
2. SEC was carried out using a SuperdexTM 30 column (self-poured SuperdexTM 30, 16 mm I.D. × 200 cm length) connected to an AKTA purifier 10 system (GE Healthcare). The flow rate was set at 0.5 ml/min with 0.5 M ammonium hydrogen carbonate and elution was monitored at $\lambda_{\text{abs}} = 232$ nm. Fractions of 1 ml were collected (typically

- sample elution occurs between 150 and 350 ml of column volume for this column). Fractions were pooled individually according to the selected partially resolved SEC peaks. The pooled fractions were then concentrated by serial cycles of lyophilization and stored at $-20\text{ }^{\circ}\text{C}$.
3. SAX-HPLC was performed on Shimadzu SCL-10AVP system equipped with a Propac PA1 column (9 mm \times 250 mm, Dionex). An analytical trial run ($\sim 50\text{ }\mu\text{g}$) was performed in order to establish where fraction collection was required, followed by preparative fractionation. About $5\text{ }\mu\text{l}$ of $0.1\text{ M NH}_4\text{HCO}_3$ was added to each tube, this amount being sufficient to neutralize 0.25 ml of elution solvent (pH 3.5). Sample (maximum 5 mg) was made up to a final volume of 0.5 ml in Milli Q H_2O and injected into the prewashed 1 ml injection loop using a Hamilton syringe. The column was prewashed for 10 min with 2 M NaCl in water, pH 3.5 (solvent B) at 1 ml/min flow rate and then equilibrated with Milli Q H_2O , pH 3.5 (solvent A) for 10 min at the same flow rate. The flow was isocratic in Milli Q H_2O (pH 3.5) for 1 min at flow rate 1 ml/min for sample loading onto the column. Samples were then eluted with a linear gradient of 0–50% solvent B (0–1 M NaCl) over 180 min. The column temperature was set at $40\text{ }^{\circ}\text{C}$ using column oven. Detection was performed online using a UV detector set at $\lambda_{\text{abs}} = 232\text{ nm}$. Fractions were collected at 15-s intervals (0.25 ml volume) using a fraction collector calibrated at 1 ml/ml flow rate, and fraction collector racks were changed at suitable time points where peak elution was absent. Fractions corresponding to the resolved or partially resolved SAX peaks were pooled. The pooled fractions were concentrated by lyophilization and stored at $-20\text{ }^{\circ}\text{C}$ (Guimond and Turnbull, 1999; Powell *et al.*, 2010).
 4. Desalting of fractions was performed on HiPrepTM 26/10 desalting column (packed with Sephadex G-25 fine) equilibrated with water on an AKTA purifier 10 system. Elution was monitored at $\lambda_{\text{abs}} = 232$ and 215 nm. The fractions corresponding to the oligosaccharide peak (which elutes first) were pooled together, after ensuring that the salt peak (which elutes later) did not overlap with the saccharide peak.
 5. Oligosaccharide concentrations were calculated by measuring the absorbance at $\lambda_{\text{abs}} = 232\text{ nm}$ and using the extinction coefficient for the double bond of $5500\text{ mol}^{-1}\text{ cm}^{-1}$ (Linhardt *et al.*, 1988).

Following library production, it is possible to screen the binding of saccharides using a variety of methods including affinity selection, ELISA assays and optical biosensors (Poplewell *et al.*, 2009; Powell *et al.*, 2004) and more recent technologies such as microarrays (Powell *et al.*, 2009; Zhi *et al.*, 2006, 2008). Saccharides can also be used for studying their effects on protein complex formation, for example, ligand–receptor interactions (Hussain *et al.*, 2006). Perhaps most important, the activity of the saccharides

can be screened in bioassays, to obtain a bioactivity profile which provides insights into actual SARs. Here, we describe an example of screening modulation of FGF signaling. Cell signaling by FGFs through FGF receptors (FGFRs) depend on HS saccharides (Rapraeger *et al.*, 1991; Yayon *et al.*, 1991). In bioassays, tissue-derived HS oligosaccharides showed diverse selectivity for both activation and inhibition of FGF signaling (Guimond and Turnbull, 1999), using cell proliferation assays with a BaF3 lymphoblastoid cell line which lacks both endogenous HS and FGFRs. These cells can be transfected with specific FGFRs to study FGF signaling (Ornitz *et al.*, 1996) and have been exploited to investigate HS saccharides function (Guimond and Turnbull, 1999; Pye *et al.*, 1998). Established transfectants which express specific FGFR isoforms can be used to screen the abilities of HS saccharides to regulate signaling; in the absence of IL-3 the cells will proliferate in response to appropriate FGFs only in the presence of exogenous activating HS oligosaccharides.

2.4.2. FGF signaling assay screening using BaF3 cells expressing FGFR1c

1. BaF3 cells transfected with FGFR1c (Ornitz *et al.*, 1996) were maintained in RPMI 1640 growth medium supplemented with 10% fetal calf serum, L-glutamine, penicillin G, streptomycin, and 1 ng/ml rmIL-3 (R&D systems).
2. FGF-1 or FGF-2 (1 nM, R&D), and selected HS saccharides are prepared in RPMI growth medium. The cells were added to the wells at final density of 10^4 – 10^5 cells/ml in the medium without IL-3, and incubated at 37 °C in 5% CO₂ for 72 h.
3. Cell viability is determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT). MTT was added for 4 h in the above conditions before solubilization by the addition of 10% SDS/0.01 M HCl for 16 h. Absorbance at λ_{abs} 570 nm is measured using a microtiter plate reader.

An example of the SAX chromatogram profile of an 8-mer fraction of porcine mucosal HS is shown in Fig. 4.3A, demonstrating a complex set of peaks from this tissue. Fractions from the start, middle, and the end of the salt gradient were selected for screening in FGF signaling assays. The selected fractions displayed variant capabilities to activate signaling by FGFR1c in response to FGF-1 (Fig. 4.3B). In addition, the BaF3 bioassay on these fractions was also performed for FGF-2 with same FGFR1c isoform, since it has been documented that FGFR1c has the same proliferative response for both FGF-1 and FGF-2 (Ornitz *et al.*, 1996). The 8-mer SAX subfractions also displayed variant activities with FGF-2, but the profile differed from that for FGF-1 (Fig. 4.3C). In particular, a number of fractions were more active than a heparin 8-mer for activation of FGF-2,

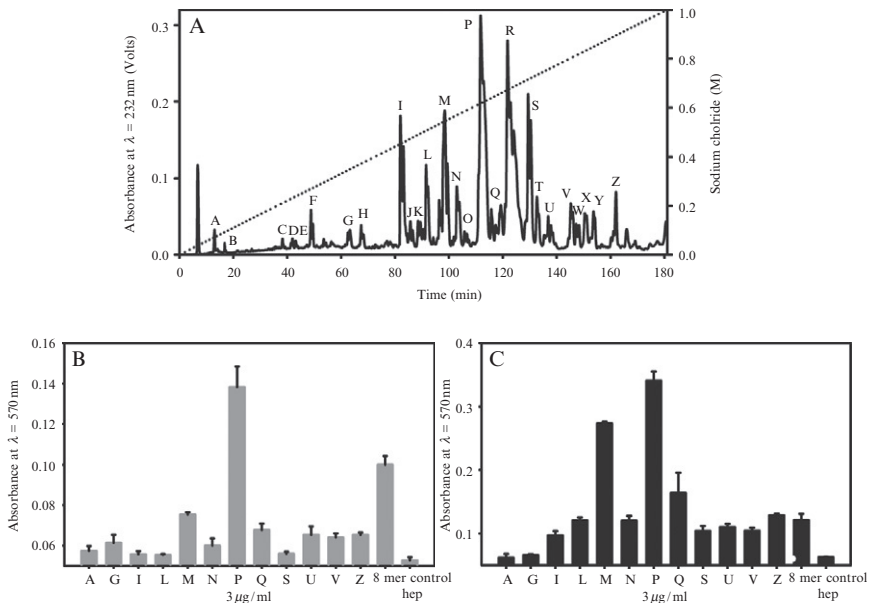


Figure 4.3 Functional profiling of HS saccharides that regulate FGF-1 and FGF-2 through FGFR1c signaling. (A) Porcine mucosal heparan sulfate 8-mer pools obtained by heparinase III digestion followed by SEC were further resolved by high resolution SAX-HPLC, performed on Propac PA1 columns (9 mm \times 250 mm) equilibrated in Milli Q H₂O, pH 3.5 at flow rate of 1 ml/min. Samples were eluted with a linear gradient of sodium chloride pH 3.5 (0–1 M over 180 min; detection by absorbance at $\lambda = 232$ nm). The figure shows a typical preparative run. Multiple separations of 5 mg per run were performed, and identical peak fractions corresponding to selected peaks were pooled and desalted on HiPrep™ desalting columns, freeze dried, and quantified. BaF3 cells were grown in the presence of (B) 1 nM FGF-1 or (C) 1 nM FGF-2. Briefly, BaF3 cells were grown in the presence of FGFs and HS 8-mer SAX-HPLC fractions at 3 μ g/ml (A, G, I, L, M, N, P, Q, S, U, V, and Z) or an 8-mer heparin saccharide; FGF with no saccharide was the control. Cell proliferation was measured at absorbance at $\lambda = 570$ nm using a 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) colorimetric assay. Values are the means of triplicate samples. Error bars represent the standard deviation, and the data are representative of two separate experiments.

whereas only fraction 8P was more active for FGF-1. These results confirmed previous report that HS saccharides differentially activate FGF signaling (Guimond and Turnbull, 1999), but indicate that the FGF-FGFR ligand activation specificities previously described in the literature (Ornitz *et al.*, 1996) will need to be revised. Finally, it is also possible to select saccharides with defined activities for sequencing (Guimond and Turnbull, 1999; Turnbull *et al.*, 1999), thus permitting detailed SARs to be explored. This is an area in which significant advances can be expected over the next few years.

3. CONCLUSIONS AND FUTURE PERSPECTIVES

The cohort of protocols described above provide one set of approaches for profiling different aspects of HS structure and activity, focusing particularly on disaccharide building blocks and larger oligosaccharide domains, the latter representing the functional units of HS chains. A range of other complementary methods in the literature have also been discussed. Taken together, these provide a new and more comprehensive toolkit to investigate HS structure and activity in a higher throughput manner in selected biological systems. They allow investigation of key questions about the actual structural variation of the heparanome in specific biological contexts and the degree of functional selectivity exerted by these structures in regulating biological processes. The extensive interactome of HS (Ori *et al.*, 2008) provides a significant glycobiology puzzle; its solution clearly requires a move toward systems biology approaches. Further advances in the application of mass spectroscopy to sequencing of HS saccharides can be expected to make a major contribution, since more complete structure–activity surveys are essential. In addition, development of databases, bioinformatics and computational biology approaches are required to automate, catalog, integrate, and model the ensuing flow of data. We anticipate that the applications of glycomics strategies over the next decade will see major advances in our understanding of the complex biology of HS.

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