Rapid purification and high sensitivity analysis of heparan sulfate from cells and tissues: towards glycomics profiling*

Scott E Guimond‡,¶, Tania M Puvirajesinghe‡,¶, Mark A. Skidmore‡, Ina Kalus§, Thomas Dierks§, Edwin A. Yates§ and Jeremy E Turnbull‡,*

‡ Centre for Glycobiology, School of Biological Sciences, The University of Liverpool, Liverpool, L69 7ZB, United Kingdom; §Department of Chemistry, Biochemistry I, Bielefeld University, 33615 Bielefeld, Germany; ¶Equal first authors.

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Address correspondence to: Prof. JE Turnbull, School of Biological Sciences, University of Liverpool, Crown Street, Liverpool, L69 7ZB. Tel: +44(0)1517954427. Email: turnbull@liverpool.ac.uk

Studies on glycosaminoglycans (GAGs) and proteoglycans (PGs) have been hampered by difficulties in isolation and analysis by traditional methods that are laborious and lack sensitivity and throughput. Here we demonstrate a simple method for Rapid Isolation of Proteoglycans (RIP) employing phenol/guanidine/chloroform reagent to purify heparan sulfate PGs (HSPGs) quantitatively from various tissues and cells. We further show that this generic purification methodology, when applied in concert with a BODIPY fluorescent label, permits structural analyses on RIP-purified HS at ~1000-fold higher sensitivity than standard UV detection methods, and ~10-100 fold higher sensitivity than previous fluorescence detection methods. The utility of RIP-BODIPY methodology was demonstrated by rapid profiling of HS structural composition from small tissue samples, multiple mouse organs, and as little as a few thousand cultured cells. It was also used to generate novel insights into in vivo structural changes in HS from Sulf1 knockout mice for the first time, that differed significantly from previous observations limited to tissue culture experiments. RIP was also applied to purify HS for bioassay testing, exemplified by cell assays of FGF signaling activation; this generated data from 2-O-sulfotransferase knockout mice, and revealed an unexpected deficiency in FGF activation by HS from heterozygous mice. These data demonstrate that RIP will underpin emerging efforts to develop glycomics profiling strategies for HS and other GAGs, to explore their structure-function relationships in complex biological systems.

Heparan sulfate (HS)1 is member of the glycosaminoglycan (GAG) family of polysaccharides and is found on almost all cell types in metazoan organisms, attached to core proteins to form specialized glycoproteins called proteoglycans (PGs). It is critical in many biological processes (including embryonic development, homeostasis, and wound healing) and lack of HS is lethal in higher organisms such as mice (1,2). HS is also involved in a variety of disease processes (such as tumor angiogenesis, pathogen adhesion and neurodegeneration). It carries out these functions primarily by binding to many different proteins and regulating their functions (1,3). Specific binding is in part determined by the variation in structure of the HS, primarily in the number and location of sulfate moieties (4,5). There is now intense interest in identifying specific structural motifs within HS responsible for binding and regulation of particular proteins, and in exploring the heparanome – the entire complements of HS structures expressed by cells and tissues (1,3,6).

The study of HS biochemistry and it’s interactions with proteins (3,4), necessitates the ability to purify and analyse HS from tissues and cells. However, current methods of purification (such as detergent and guanidium salt extraction followed by protease digestion, or chloroform/methanol extraction) whilst good at purifying HS in large amounts from single sources, suffer several drawbacks. They are lengthy and laborious (involving complex extraction processes and multiple column chromatographic steps) and can also result in alteration of native structure, for example de-N-sulfation (7-10). To
address these problems our rationale was to devise a simple extraction and purification protocol which was both rapid and streamlined, minimizing transfers to reduce losses in yield, and providing HS of sufficient purity for comparative structural and functional analysis. We observed that PGs partition exclusively in the aqueous phase in extractions performed with TRIzol® (a well known phenol/guanidine/chloroform reagent that is widely used to purify DNA and RNA from tissues) (11). This observation led us to develop a method for the rapid isolation of proteoglycans (RIP) from most tissues and cell culture samples that is quick (from cell/tissue sample to ion exchange purification in ~30 min), reduces loss of material (only one transfer before ion exchange step), and is readily scalable. Furthermore, when coupled to a recently developed method for highly sensitive fluorescent labeling of GAG saccharides with BODIPY hydrazide (12), RIP allows the structural profiling and bioassay of HS from less than a milligram of starting tissue or a few thousand cells.

**EXPERIMENTAL PROCEDURES**

**Materials:** Chloroform, DNase I, RNase, neuraminidase, chondroitin ABC lyase, sodium hydroxide, sodium borohydride, sodium chloride, tris acetate, sodium acetate, calcium acetate, PBS, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), SDS and DMSO were purchased from Sigma Aldrich (Dorset, UK); TRIzol®, DMEM, RPMI-1640, fetal calf serum, L-glutamine, penicillin-G, streptomycin sulfate, and BODIPY were from Invitrogen (Paisley, UK); Recombinant murine IL-3 and recombinant human FGF-2 from R&D Systems (Abingdon, UK); porcine intestinal mucosa heparin from Celsus Labs (USA); DEAE-Sephacel and PD-10 columns from GE Healthcare (Buckinghamshire, UK); heparinas I, II, III from IBEX Technologies (Montreal, Canada); Pronase from Roche (Welwyn, UK); HPLC-grade water and methanol from VWR (Lutterworth, UK); and unsaturated heparin disaccharide standards from Dextra Labs (Reading, UK). BaF3 cells expressing FGFR1c were generously provided by David Ornitz (Washington University, St. Louis, USA).

**Tissues:** Organs from normal adult female CD1 mice were obtained from the University of Liverpool Animal House. Sulf1 knockout mice were generated as previously (13,22). For cerebella preparation, heterozygous littermates were intercrossed and after genotyping cerebella were dissected from newborns at postnatal day 6. Mouse brains from mice wild-type, heterozygous or null for 2-O sulfotransferase (14) were a gift from Dr Val Wilson, Edinburgh.

**3T3 cell culture:** Murine Swiss 3T3 fibroblasts (ATCC# CCL-92) were maintained in DMEM supplemented with 10% fetal calf serum, 4 mM L-glutamine, 10 µM penicillin G and 10 µM streptomycin sulfate. Cells were allowed to reach 10,000 cells/cm² in 100 mm dishes, 25 mm dishes and 24 well plates prior to extraction. Cell numbers were obtained by counting duplicate plates.

** Extraction of HSPGs with TRIzol®:** The extraction of HSPGs is identical to the initial purification procedure for RNA. Briefly, wet tissue or cultured cells were homogenized in a tissue grinder or solubilized in the culture dish with 1 ml of TRIzol® reagent per 100 mg tissue or 10cm² culture dish. Samples were then incubated for 5 minutes at room temperature and transferred to polypropylene tubes with the addition of 0.2 ml of chloroform per 1 ml original TRIzol® reagent used. Tubes were shaken vigorously for 15 seconds and incubated a further 3 minutes at room temperature. Samples were centrifuged at ~8000xg for 15 minutes at 4°C. HSPGs are present in the upper, aqueous phase (Fig 1). The aqueous phase was applied to a 0.1 to 1 ml DEAE column (~0.1 ml of bed/ml sample) in a disposable mini-column. The column was washed with 10 vol. PBS pH 7.4, 10 vol PBS (0.25 M NaCl) pH 7.4 and eluted with 10 vol PBS (2M NaCl) pH 7.4. PBS (2M NaCl) fractions were desalted on PD10 gel filtration columns and freeze dried (“crude” GAG fraction, see Fig 1).

**Additional HS purification:** Freeze dried samples were reconstituted in 100 µl of HPLC-grade water and digested in a single tube by sequential addition and incubation at 37°C with 2.5 mM chondroitin ABC lyase (in 500 mM tris acetate pH 8.0; 4 hrs), 10 mM neuraminidase (in 125 mM sodium acetate pH 5.0; 4 hrs) and 2 mg/ml Pronase (in 500 mM tris acetate, 50 mM calcium acetate; 16 hrs). Each enzyme and buffer (at 5x final concentrations) were added at 1/5 volume at each step. The mixture was then subjected to DEAE chromatography and desalting as described above. Finally, the samples were applied to Millipore Biomax centrifuge filters (5K NMWL, Millipore, Watford, UK) and centrifuged for 5 minutes at 5000 rpm. The retentate (50 µl) was brought to 500 µl with HPLC-grade H₂O.
and reapplied to the centrifuge filters. This process was repeated 3 times to ensure remove of salts and other low molecular weight contaminants. The final retentate (purified GAG fraction, see Fig 1) was stored at -20°C until use.

**Disaccharide analysis with UV detection:** Samples in 100 mM sodium acetate, 0.1 mM calcium acetate, pH 7.0 were digested by sequential addition at 37°C of 2.5 mU heparinase I (4 hrs), followed by 2.5 mU of heparinase III (4 hrs) and finally with 2.5 mU of heparinase II (16 hrs). Samples were then applied to a Propac PA-1 strong-anion exchange column (4.6mmx250mm; Dionex, Leeds, UK) and eluted with a 0-1M NaCl gradient over 45 minutes on a Dionex BioLC HPLC at a flow rate of 1ml/min. Disaccharides were identified with reference to authentic heparin unsaturated disaccharide standards.

**Determination of HS concentration:** The amounts of HS present in samples was determined by digesting the purified HS to completion as described above for disaccharide analysis. The molar concentration of the disaccharides present in solution was determined at A 232 using the Beer-Lambert Law with an extinction coefficient of 5500 mol⁻¹ cm⁻¹ for the unsaturated bond chromophore generated by heparinase enzymes.

**BODIPY hydrazide labeling and HPLC analysis with fluorescence detection:** Disaccharides obtained as for UV detection were labeled with BODIPY hydrazide, as previously described (12). Briefly, lyophilized samples were suspended in BODIPY™ FL hydrazide (5mg/ml in methanol;10µl). Methanol was removed by centrifugation under vacuum and samples were re-suspended in DMSO:ethanoic acid (17:3 v/v; 10µl) prior to incubation for 4 hrs at room temperature in the dark. Labeled samples were reduced using sodium borohydride (1M, aqueous; 10µl) and incubated for 30 mins (r.t.) prior to flash-freezing in liquid nitrogen and lyophilisation. The fluorescently labelled disaccharides were resuspended in DMSO:H₂O (50:50 v/v, 1ml) prior to loading onto a Propac PA-1 column as above, eluted using a linear gradient of 0-1M sodium chloride (in 150mM NaOH) over 30mins at a flow rate of 2ml/min on a Shimadzu HPLC system. Peaks were detected using inline fluorescence detection at excitation wavelength of λ=488nm and an emission wavelength of λ=520nm using a Shimadzu RF-551 detector. The column was reconditioned by washing with 2M NaCl (in 300mM NaOH) before equilibrating in 150mM NaOH. Previously calculated correction factors were applied to quantitate the observed disaccharides (12).

**BaF3 assay:** BaF3 cells (naturally devoid of HS and FGF receptors) transfected with FGFR1c were maintained as described previously (15). For proliferation assays, 10,000 cells/well were plated on a 96-well plate with culture medium (100 µl, RPMI-1640 supplemented with 10 % foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin-G and 100 µg/ml streptomycin sulfate without IL-3) and were incubated with 2 ng/ml IL-3 or 1 nM FGF-2 with and without heparin/HS at the concentrations indicated. After 72 h at 37 °C, 250 µg/ml MTT was added and the cells were incubated a further 4 hours at 37 °C. Following solubilization in 10% SDS, 0.01N HCl the plates were read on a Multiscan EX spectrophotometric plate reader (Thermo Electron, Basingstoke, UK) at 570 nm. Increase in absorbance is proportional to cell number.

**RESULTS**

**Rapid method for HSPG extraction and purification**

In order to streamline purification of HS from tissues and cell culture, we examined the benefits of a strategy exploiting phenol/guanidine/chloroform reagent (eg. the commercial reagent TRIzol®) (Fig 1). We observed that GAGs (including HS) appear exclusively in the aqueous layer after phase separation. Following this initial isolation step, the HS is subjected to routine anion exchange chromatography on a mini-column, and desalted, resulting (typically after ~ 2 hours) in a crude GAG preparation which can be used for initial structural analysis (Fig 1). Further purification was achieved by sequential single-pot enzyme digestion and a second anion exchange/desalting procedure to remove unwanted GAGs and contaminating proteins. This results (after ~ 24 hours) in highly purified HS preparation suitable for detailed structural profiling and also bioassays (Fig 1).

A systematic approach was adopted to determine the efficacy of the RIP method in terms of recoveries (spiking experiments with ³H-labelled HSPGs) and maintenance of structural integrity. We examined the efficacy of the RIP method of purification in terms of yields. Initially ³H-labeled syndecan-1 was spiked into 100 mg of fresh mouse brain homogenate and HS purified by RIP. The amount of ³H label in the organic phase, interphase and aqueous phase of the
TRIzol® extract were determined and showed that >95% of the spiked 3H-labeled HSPG was recovered in the aqueous phase (Fig. 2). Similar results were obtained by comparing yields of spiked samples from liver extracts (data not shown). It is well known that HS can be N-desulfated when exposed to low pH conditions (7-10). Since TRIzol® has a pH of around 4.0 it was necessary to determine whether HS underwent any desulfation during the RIP procedure. Heparin, a highly sulfated variant of HS, was used as a proxy molecule in these control experiments, and was subjected to RIP purification followed by 13C NMR analysis (10). The resulting NMR spectra confirmed that the purified heparin was identical to parental (untreated) heparin (data not shown), confirming that the procedure does not structurally modify these polysaccharides.

**Structural Profiling of HS from Tissues by Fluorescence Detection**

We next exploited the RIP method by coupling it with a newly developed method for reducing-end labeling of GAG saccharides with the fluorescent tag BODIPY hydrazide (12). With a detection limit of ~100 fmol, this provides a >1000-fold increase in sensitivity over the use of UV absorbance (A_232), commonly used to detect the unsaturated non-reducing end bond produced by the action of the heparin lyase enzymes (16)) and a 10 to 100-fold increase in sensitivity over previous fluorescent labels (17-20). Fluorescence also has the advantage of minimising background noise and non-glycan peaks, thus enhancing sensitivity. HS isolated from mouse kidney was subjected to exhaustive heparinase digestion and the resulting disaccharides were labeled with BODIPY hydrazide and separated by high resolution strong anion exchange HPLC (Fig 3A). The results show a typical chromatogram for HS, with a high proportion of unsulfated disaccharide (UA-GlcNAc) and smaller percentages of the other common disaccharides. Peaks eluting before 5 minutes represent free tag (see Fig 4); importantly, since no labelled saccharides co-elute with free tag, the samples do not require clean up to remove this before analysis, thus streamlining analysis.

To determine the minimum amount of starting material required to obtain accurate structural data, we extracted HS from 100 mg, 10 mg and 1 mg of mouse kidney (wet weight) and undertook comparative disaccharide analysis using BODIPY hydrazide (Fig 3). All three samples gave comparable results, indicating the effectiveness and accuracy of the RIP-BODIPY method. The signal obtained from 500 µg of tissue was still above the detection limits, so it should in principle be possible to analyse smaller amounts. This confirms that minute quantities of tissue can be used for accurate structural profiling of HS.

In order to demonstrate the profiling potential of this methodology, we repeated the RIP extraction and BODIPY structural analysis of a range of mouse organs (Fig 5). Liver, lung, spleen and heart were all analyzed and compared with the kidney, along with a comparison between fluorescence and standard UV detection (Fig 6 and Table 1). Each tissue yielded a unique compositional profile, with heart and kidney being relatively low sulfated and liver and lung being more highly sulfated. Although the UV data was generally in very close agreement with that obtained by BODIPY detection, some differences in quantitation between these two modes of detection were noted (Table 1), as seen in previous studies (20). The data were also in broad agreement with previous tissue HS data obtained using standard purification and analysis techniques including UV detection or post-column fluorescence labelling (20-22). In addition, data on the disaccharide composition of porcine mucosal heparin obtained by BODIPY detection was comparable to that obtained by UV detection data and also literature values (data not shown). Taken together this provides validation of the RIP-BODIPY methodology.

**Structural Profiling of HS from Tissue-culture Cells by Fluorescence Detection**

We next tested the application of the RIP method to cells grown in culture. HS was purified from Swiss 3T3 cells by RIP and analysed by BODIPY labeling and SAX HPLC. HS purified from 3T3 cells grown in 100 mm dishes (250,000 cells), 35 mm dishes (40,000 cells) or 24 well plates (4,000 cells) produced comparable results, indicating that the methodology permits profiling from as little as a few thousand cells (Fig 7). Interestingly, the HS from 3T3 cells has a higher proportion of di- and trisulfated disaccharides (standards 5, 6 and 8) than the mouse tissues (Fig 6 and Table 1). These data demonstrate the utility of the methodology for profiling HS structures from small numbers of tissue culture cells.
RIP-BODIPY Analysis of HS from Sulf1 Knockout Mice

A major advantage of the new methodology is application in systems that have previously been intractable due to lack of sufficient sample, such as knockout mouse models with altered HS molecular phenotypes, exemplified by the Sulf family of enzymes. These are glucosamine endosulfatases that remove 6-O-sulfates from HS in the extracellular environment (23). This activity is considered crucial for the proper regulation of several growth factors (25). Mice deficient in Sulf activity show a number of specific developmental defects, which become most obvious for the brain (25). A lack of Sulf activity would be expected to result in HS with more 6-sulfates than wild type mice. This has been observed in Sulf knockout mouse embryonic fibroblasts grown in vitro (23,26) but has not yet been studied in vivo. However, it is known that HS biosynthesis is dynamic, and alters when cells are placed in culture conditions (27-29). This leaves open the question as to whether the in vivo data truly reflects the in vitro data.

To explore this issue we analysed individual cerebella (average weight 26.8 ± 4.3 mg) from postnatal day 6 Sulf-1 knockout mice, using the RIP-BODIPY methodology (Fig 8). We found that the cerebellar HS from Sulf1 null mice displayed significantly higher proportions of each of the specific 6-O-sulfate containing disaccharides (2, UA-GlcNAc(6S); 4, UA-GlcNS(6S); 6, UA(2S)-GlcNS(6S) and 8, UA(2S)-GlcNAc(6S)) than wild type littermates (Fig 8C). However, the increase was most significant for disaccharides 6 and 8, and an increase in a non-6-O-sulfated disaccharide 5 (UA(2S)-GlcNS) was also notable. The RIP-BODIPY methodology was also used to demonstrate differences in HS structure between Sulf-1 and Sulf-2 knockout mice (see Kalus et al, manuscript submitted to J Biol Chem; ID#: JBC/2009/028233). This data represents the first measurement of in vivo changes in the HS structural profile in these knockout mice, and demonstrate the potential for RIP-BODIPY to be used for glycomics profiling of HS in function-specific contexts in animal models.

Bioassay of HS purified from mouse tissue by RIP

A further advantage of RIP is rapid purification of HS samples for bioassays. To demonstrate this we studied mice with mutations in the HS biosynthetic enzyme 2-O sulfotransferase (2OST); the null genotype results in early postnatal lethality due to lack of kidneys (14) and also defects in neural development (30). Embryonic mice fibroblasts lacking this enzyme activity have altered HS structure with essentially no 2-O sulfation and a compensatory increase in 6-O and N-sulfation (31). To explore the functional effects of these changes in HS structure, we purified HS by RIP from the brains of mice that were null, heterozygous and wild type for the 2OST gene, and tested the samples in HS-dependent bioassays of FGF signaling in HS-dependent BaF3 lymphoid cell lines (15). HS purified by RIP from wild type mice supported FGF-2 signalling via FGFR1 (Fig 9); in marked contrast, HS from null mice lacked any ability to activate FGF2-FGFR1 signaling (Fig 6), as predicted from the critical role of 2-O-sulfate groups in HS binding to FGF2 (32). Unexpectedly however, we also noted that HS from heterozygous mice also lacked activity (Fig 9), revealing for the first time that gene dosage is critical for maintaining correct 2OST action in HS biosynthesis to create functional HS sequences for FGF2 signalling via FGFR1.

DISCUSSION

Purification and analysis of specific structures from HS that relate to particular biological functions is a significant challenge, yet remains a requirement to identify HS structures in cell culture and in vivo that have specific activities. Given the low abundance of specific structures and the pressing need for higher throughput glycomics studies, rapid scalable methods that minimize sample loss and are compatible with sensitive detection are now essential. Here we have described a new method for HS GAG purification – RIP (Fig 1) - which is compatible with many different types of tissue (Fig 5) and cells (Fig 7). Furthermore, using BODIPY labeling and fluorescence detection with a standard HPLC fluorimeter provides a 10- to 100-fold increase in sensitivity (8) over previous fluorescent methods (10-13), and permits structural profiling data on HS to be generated with less than a milligram of starting material (Fig 5), or as little as a few thousand cultured cells (Fig 7). This means that new biological systems can be accessed for the first time. In addition, the streamlined nature of the protocol increases the number of samples that can be purified at one time, providing high throughput potential for the first time for these molecules. Data obtained is comparable to previously used standard methods (20-22). Interestingly, we have noted some
differences in quantitation between UV and fluorescence detection, as noted previously by other investigators (20). Assuming careful experimental procedures and application of appropriate labeling correction factors, these differences may reflect more accurate measurement of compositional analysis by fluorescence detection due to less inherent susceptibility to sample contamination and background noise (see legend, Table 1). The latter are a significant limitation for UV detection, especially where only small amounts of sample are available.

Here we demonstrated that purification of HS using RIP and subsequent disaccharide profiling with BODIPY detection allowed us to detect structural changes in HS from single postnatal cerebella of mice mutant in Sulf-1 (Fig 8). These changes suggest that Sulf 1 normally removes 6-O-sulfates from all potential 6-O-sulfated disaccharides, but most notably from trisulfated disaccharides [UA(2S)-GlcNS(6S)]; interestingly, this action pattern of Sulf-1 differs significantly from that observed in fibroblast cells derived from Sulf-1 knockout animals and cultured in vitro. In the latter case, an increase in all 6-O-sulfated disaccharides was also observed (23), but with far less pronounced effects than those observed here for the in vivo analysis of cerebellum tissue, and in different relative ratios. This is likely due to altered biosynthesis induced by placing the cells in culture conditions (29), and emphasizes the importance of studying HS structures from in vivo tissues in order to accurately decode the true structure-function relationships of HS. The application of the RIP-BODIPY methodology was also exploited to compare HS from Sulf-1 and Sulf-2 knockout mice; clear differences in the HS molecular phenotype were revealed which suggest divergent action of these enzymes in vivo (see Kalus et al, manuscript submitted to J Biol Chem; ID#: JBC/2009/028233). This data represents the first measurement of in vivo changes in the HS structural profile in these knockout mice, and demonstrate the potential for RIP-BODIPY to be used for glycomics profiling of HS in function-specific contexts in animal models.

It should also prove possible to exploit and adapt the RIP-BODIPY methodology for both oligosaccharide mapping of HS domain structures (33), and for integral glycan sequencing (34), probably using HPLC (15) or capillary electrophoresis separation. Furthermore, the sensitivity of BODIPY detection can potentially be further increased (~100-1000 fold) using laser-induced fluorescence detection (35), allowing even smaller amounts of tissue or cells to be analysed in the future.

RIP purification does not alter the structure of the HS and the HS that is purified can also be used in bioassays (Fig 9), allowing structure-activity relationships to be explored directly, and providing insights into the functional consequences of altered HS molecular phenotypes. Here we demonstrated differences in the bioactivity of HS from mice with variant genotypes for HS 2-O sulfotransferase. While HS from wild type mice activates FGF-2 signalling through FGFR1, HS from null mice cannot (Fig 9), in agreement with previous data on FGF2 stimulation of embryonic fibroblasts from these mice (31). This is not unexpected, since 2-O sulfates have been shown to be important for the binding of FGF-2 to heparin (32). Surprisingly however, our new data revealed that HS from heterozygous mice was also unable to activate FGF-2 signalling, indicating the importance of gene dosage for correct 2OST action in HS biosynthesis (Fig 6). Although HS from heterozygous mice has 2-O-sulfates, changes in the fine structure of HS are known to affect its biological activity and presumably underly this lack of activity (15). Thus, RIP methodology provides an important additional tool for tackling the complexities of HS structure-activity relationships from in vivo tissue samples.

In summary, the RIP method opens up new avenues for studying specific HS and other GAG structures present in cells or tissues despite their low abundance when coupled with high sensitivity techniques such as fluorescent labeling (12). The application of RIP should enhance development of glycomics approaches for GAGs (36,37), including emerging mass spectrometry-based approaches (38), and will undoubtedly bring progress in uncovering the in vivo structure-function relationships of the heparanome (1,3,6) in complex biological systems.
REFERENCES


FOOTNOTES

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The abbreviations used are: HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; GAG, glycosaminoglycan; RIP, rapid isolation of proteoglycan; PG, proteoglycan; Sulf, sulfatase.

FIGURE LEGENDS:

**Figure 1:** RIP strategy for the isolation of HS from tissues and cells. Cells or animal tissues are subjected to homogenization in phenol/guanidine reagent, and PGs partition into the aqueous phase after chloroform addition. This process is rapid (~20 min) compared to traditional methods (~48 hrs). PGs are then partially purified using DEAE ion exchange chromatography and desalted, yielding crude GAG extract (~ 2-3hrs) suitable for initial structural analysis. Contaminating macromolecules are removed by single-pot sequential enzymatic digests, DEAE chromatography, desalting and filtration. This yields a highly purified GAG sample (~ 24hrs) for use in bioassays and structural profiling.

**Figure 2:** 3H-labeled proteoglycan partitions in the aqueous phase after extraction in Trizol. 3H-labeled syndecan-1 was spiked into 100 mg of fresh mouse brain homogenate and extracted in Trizol. The organic, interphase and aqueous phase were separated and the amount of 3H in each was determined using a Wallac WinSpectra 1414 Liquid Scintillation Counter and Optiphase HiSafe 3 Scintillant (Perkin-Elmer, Beaconsfield, UK). Data are expressed as the DPM in each phase (mean +/- SD, n=3).

**Figure 3:** Structural profiling of disaccharides derived from RIP-extracted tissue HS using fluorescence detection. HS was purified from differing amounts of mouse kidney by RIP and disaccharide compositions profiled using HPLC and BODIPY fluorescence detection. Representative profiles are shown for data obtained from (a) 100 mg, (b) 10 mg and (c) 1 mg of starting material (analysis was performed on ½ of each sample). Elution positions of the 8 commonly occurring disaccharides are indicated. Panel d, quantitative comparison of the compositional profiles expressed as % total disaccharides (mean +/- sd; N=3). Disaccharide standards: 1, UA-GlcNAc; 2, UA-GlcNAc(6S); 3, UA-GlcNS; 4, UA-GlcNS(6S); 5, UA(2S)-GlcNS; 6, UA(2S)-GlcNS(6S); 7, UA(2S)-GlcNAc; 8, UA(2S)-GlcNAc(6S).

**Figure 4:** Expanded chromatogram of BODIPY labeled mouse kidney HS disaccharides showing early-eluting free tag peaks. HS was purified from 1 mg of mouse kidney by RIP, digested with heparinases, labeled with BODIPY hydrazide and the disaccharides separated by HPLC as described in Methods. HPLC analysis was performed on ½ of the extracted sample. Free tag elutes before 10 minutes under the wash conditions used and thus does not interfere with clear measurement of the disaccharides.

**Figure 5:** Structural profiling of HS from different mouse tissues using RIP extraction and fluorescence detection. HS was purified from 100 mg of each tissue by RIP, digested with heparinases, labeled with BODIPY hydrazide and separated by HPLC as described in Methods. Representative data is shown for the tissues: (a) liver, (b) lung. Disaccharide standards were as described in Fig 3. Panel (c) shows a quantitative comparison of the compositional profiles (data are expressed % of total disaccharide; mean +/- SD, n=5). Data from the full panel of tissues is provided in Fig 6 and Table 1.

**Figure 6:** Comparison of UV vs Fluorescent disaccharide analysis of RIP-extracted HS from multiple mouse tissues. Bar chart of data in Table 1. HS was purified by RIP from 100 mg of each tissue by RIP, digested with heparinases, and disaccharide composition measured by HPLC with UV or fluorescence detection (in the latter case after BODIPY hydrazide labeling) as described in Methods. Quantitative comparisons of the compositional profiles are shown for kidney, liver, spleen, lung and heart (data are expressed % of total disaccharide; mean +/- SD, n=5). Disaccharide standards were as described in Fig 3.

**Figure 7:** Structural profiling of HS extracted from Swiss 3T3 fibroblasts. HS was purified from Swiss 3T3 cells by RIP, digested with heparinases, labeled with BODIPY hydrazide and separated by HPLC as described in Methods. The whole of each extracted sample was run and representative data are shown for (a) 250,000 cells;
(b) 40,000 cells (c) 4,000 cells. Panel (d) shows a quantitative comparison of the compositional profiles (expressed as % of total disaccharide; mean +/- SD; n=3). Disaccharide standards were as described in Fig 3.

**Figure 8: Increase in 6-O-sulfate containing disaccharides in the cerebellum of postnatal day 6 mice deficient in Sulf 1.** HS was purified from single postnatal day 6 mouse cerebella from Sulf1 knockout and wild type mice by RIP, digested with heparinases, labeled with BODIPY hydrazide and separated by HPLC as described in Methods. Representative data was obtained for (a) Sulf1 +/+ mice and (b) Sulf1 +/- mice. Panel (c) shows a quantitative comparison of the compositional profiles (expressed as % of total disaccharide; mean +/- SD; n=3). Disaccharide standards were as described in Fig 3. Inset: expansion of y-axis showing detail of disaccharides 5-8.

**Figure 9: Bioactivity of HS purified from brains of mice deficient in 2-O sulfotransferase.** BaF3 lymphoid cells expressing FGFR1c were incubated with 1 nM FGF-2 for 72 hours in the presence of increasing concentrations of heparin, or HS purified by RIP (from the brains of mice wild type, heterozygous or null for 2-O sulfotransferase activity). Proliferation was quantified as described in Methods (mean +/- SD; n=3).
Table 1: Comparison of UV vs Fluorescent disaccharide analysis of RIP-extracted HS from multiple mouse tissues

HS was purified from 100 mg of starting material, digested with heparitinases, labeled with BODIPY hydrazide and run on HPLC as described in Methods. Data are expressed % of total disaccharide (mean +/- SD, n=5). It should be noted that the small differences in the amounts of specific disaccharides detected using BODIPY vs. UV are likely related to small differences in the extinction coefficients of the non-reducing end double bond caused by differences in structure (39) In contrast the BODIPY-labelled saccharides are quantitated with a correction factor for variations in reducing end labeling efficiencies based on chemical quantities, based on studies with authentic disaccharide standards (12). UV data is also subject to variable differences in the accuracy of subtraction of background noise from peaks, particularly so in the case of minor peaks and where sample amounts are low. There are often high levels of contaminating buffer or sample peaks in the earlier parts of the chromatogram, combined with general baseline drift. This results in a tendency to over-estimate the amounts of lower sulfated disaccharides, and under-estimate the amounts of the higher sulfated or low abundance disaccharides. Fluorescence chromatograms are generally much less susceptible to these effects.

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>Kidney</th>
<th>Liver</th>
<th>Spleen</th>
<th>Lung</th>
<th>Heart</th>
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<td>56.58 (2.26)</td>
<td>51.20 (5.83)</td>
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<tr>
<td>2 UA-GlcNAc(6S)</td>
<td>UV</td>
<td>6.61 (0.85)</td>
<td>4.86 (0.9)</td>
<td>10.13 (0.97)</td>
<td>10.96 (0.32)</td>
</tr>
<tr>
<td></td>
<td>Fluorescence</td>
<td>8.91 (0.27)</td>
<td>3.96 (0.08)</td>
<td>9.52 (9.52)</td>
<td>11.48 (0.46)</td>
</tr>
<tr>
<td>3 UA-GlcNS</td>
<td>UV</td>
<td>19.42 (3.55)</td>
<td>18.19 (1.45)</td>
<td>18.37 (1.77)</td>
<td>20.45 (4.11)</td>
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<tr>
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<td>Fluorescence</td>
<td>14.47 (0.43)</td>
<td>18.11 (0.36)</td>
<td>17.75 (3.55)</td>
<td>21.15 (0.21)</td>
</tr>
<tr>
<td>4 UA-GlcNS(6S)</td>
<td>UV</td>
<td>7.35 (0.13)</td>
<td>7.01 (1.07)</td>
<td>10.36 (0.21)</td>
<td>2.41 (0.35)</td>
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<tr>
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<td>Fluorescence</td>
<td>8.38 (0.17)</td>
<td>6.84 (0.14)</td>
<td>9.30 (0.09)</td>
<td>1.20 (0.16)</td>
</tr>
<tr>
<td>5 UA(2S)- GlcNS</td>
<td>UV</td>
<td>4.27 (0.13)</td>
<td>6.73 (0.69)</td>
<td>2.09 (0.1)</td>
<td>9.94 (0.27)</td>
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<tr>
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<td>Fluorescence</td>
<td>6.18 (0.19)</td>
<td>5.67 (0.11)</td>
<td>3.58 (2.18)</td>
<td>9.83 (1.28)</td>
</tr>
<tr>
<td>6 UA(2S)- GlcNS(6S)</td>
<td>UV</td>
<td>2.12 (0.41)</td>
<td>8.05 (1.07)</td>
<td>1.02 (0.93)</td>
<td>3.06 (0.72)</td>
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<tr>
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<td>Fluorescence</td>
<td>5.59 (0.56)</td>
<td>11.41 (1.6)</td>
<td>1.36 (0.12)</td>
<td>3.33 (0.43)</td>
</tr>
<tr>
<td>7 UA-2S-GlcNAc</td>
<td>UV</td>
<td>2.87 (0.76)</td>
<td>1.79 (0.31)</td>
<td>1.40 (0.76)</td>
<td>3.47 (0.82)</td>
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<tr>
<td></td>
<td>Fluorescence</td>
<td>3.16 (0.73)</td>
<td>1.05 (0.02)</td>
<td>1.90 (2.43)</td>
<td>1.81 (0.36)</td>
</tr>
<tr>
<td>8 UA-2S-GlcNAc(6S)</td>
<td>UV</td>
<td>1.03 (0.24)</td>
<td>1.12 (0.30)</td>
<td>0.3 (0.05)</td>
<td>ND (0)</td>
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<tr>
<td></td>
<td>Fluorescence</td>
<td>1.24 (0.50)</td>
<td>1.61 (0.03)</td>
<td>0.01 (0.01)</td>
<td>ND (0)</td>
</tr>
</tbody>
</table>
Figure 1

Homogenisation + phenol/guanidine + chloroform

Aqueous phase

<table>
<thead>
<tr>
<th>Ion exchange chromatography</th>
</tr>
</thead>
</table>

Bound material eluted with high salt wash

Desalt (FFLO) and freeze dry

Salt free sample

Crude GAG extract

<table>
<thead>
<tr>
<th>Sample clean up using enzymes</th>
</tr>
</thead>
</table>

DEAE, Desalt

Structural analysis→ Purified GAG → Bioassays
Figure 2

![Graph showing DPM values for Organic, Interphase, and Aquous phases.]

- DPM values range from 0 to 50000.
- Organic phase has the lowest DPM values.
- Interphase phase has moderate DPM values.
- Aquous phase has the highest DPM values.
Figure 3

(a) Graph showing fluorescence intensity over time (Minutes).
(b) Graph with similar data as (a).
(c) Graph with fluorescence intensity over time (Minutes).
(d) Bar chart showing % total disaccharide by various disaccharides and different concentrations (100 mg, 10 mg, 1 mg).
Figure 5

(a) Fluorescence intensity over time (minutes)

(b) Fluorescence intensity over time (minutes)

(c) % Total Disaccharide by Liver and Lung
Figure 6

Disaccharide % Total Disaccharide

Kidney UV Fluorescence

Liver UV Fluorescence

Spleen UV Fluorescence

Lung UV Fluorescence

Heart UV Fluorescence
Figure 7
Figure 9

![Graph showing MTT assay (A570) vs. Saccharide concentration (ng/ml) for different samples: Heparin, 2OST Wild type, 2OST Het, 2OST Null.](http://www.jbc.org/)
Rapid purification and high sensitivity analysis of heparan sulfate from cells and tissues: towards glycomics profiling
Scott E. Guimond, Tania M. Puvirajesinghe, Mark A. Skidmore, Ina Kalus, Thomas Dierks, Edwin A. Yates and Jeremy E. Turnbull

J. Biol. Chem. published online July 13, 2009

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