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Enabling Methodology for the End Functionalisation of Glycosaminoglycan Oligosaccharides**†

Emiliano Gemma,[1] Odile Meyer,[1] Dušan Uhrín[1] and Alison N. Hulme[1,*]

[1]EaStCHEM, School of Chemistry, Joseph Black Building, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JJ, UK.

[*]Corresponding author; e-mail: Alison.Hulme@ed.ac.uk, tel.: + 44 131 650 4711, fax: + 44 131 650 4743

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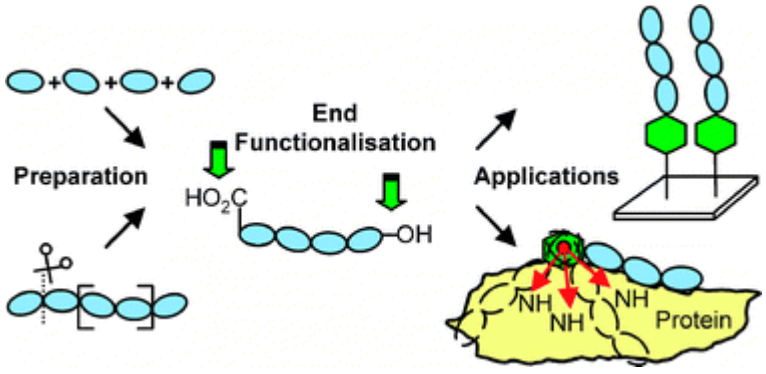
Abbreviations:
Abbreviations used in this review, are as follows: GlcNAc = *N*-acetyl-D-glucosamine, GalNAc = *N*-acetyl-D-galactosamine, GlcA = D-glucuronic acid, IdoA = L-iduronic acid, ΔUA = 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid, GlcNS = *N*-sulfo-D-glucosamine, GlcNS(6S) = *N*-sulfo-6-*O*-sulfo-D-glucosamine, IdoA(2S) = 2-*O*-sulfo-L-iduronic acid, ΔUA(2S) = 4-deoxy-2-*O*-sulfo-L-*threo*-hex-4-enopyranosyluronic acid.

Graphical abstract:



Abstract

The chemical functionalisation of glycosaminoglycans is very challenging due to their structural heterogeneity and polyanionic character; but as an enabling technology it promises rich rewards in terms of the structural and biological data it will afford. This review surveys the known methods for the preparation of glycosaminoglycan oligosaccharides and conditions for the selective functionalisation of both the reducing and non-reducing ends. The synthetic merits of each approach are discussed, together with the structural modification of the glycosaminoglycan oligosaccharide which they confer. Recent applications of this methodology are highlighted, including introduction of functional labels for gel mobility shift assays and NMR studies of glycosaminoglycan-protein complexes, and synthesis of immobilised glycosaminoglycan arrays

1. Introduction

Glycosaminoglycans (GAGs) are linear polysaccharides found almost ubiquitously on animal cell surfaces and within extracellular matrices.[1] They constitute an important class of macromolecules that are implicated in both the structural organisation of extracellular matrices, and how cells interact with them, as well as in the regulation of the biological activity of morphogens, growth factors, cytokines, chemokines and enzymes,[2,3] and regulation of the immune system.[4,5] Many of the functions of GAGs are mediated through their interactions with proteins;[6] which occur via contacts between the negatively charged groups of GAG oligosaccharides and positively charged amino acid side chains. As a consequence, oligosaccharides in protein-GAG complexes do not occupy hydrophobic pockets, but sit on the protein surface with only a few intermolecular contacts,[7,8] making solution phase structure determination of these complexes very challenging. Despite a wealth of chemical and genetic evidence to suggest that control of the fine structure of GAGs including their detailed sulfation patterns is crucial for their function *in vivo*,[9-12] there have been comparatively few studies of these interactions at the molecular level due to a lack of enabling chemical tools, and even fewer studies where these interactions are placed in a broader context as part of the emerging field of glycomics.[13]

The four basic classes of GAGs [heparin/heparan sulfate (HS), chondroitin sulfate (CS)/ dermatan sulfate (DS), keratan sulfate (KS) and hyaluronan (HA)] are produced by a common biosynthetic pathway in which the linear alternating uronic acid/hexosamine polymeric chain is extended in a stepwise fashion.[14] Heparin/HS, CS and DS share a common tetrasaccharide motif that links the GAG chain to the protein core.[10] Coupling of either UDP-GlcNAc [heparin/HS] or UDP-GalNAc [CS, DS] to the proteoglycan core, differentiates the developing GAG;[10] this is followed by coupling of UDP-gluronic acid (GlcA) to the hexosamine residue [either (1→3) or (1→4)

dependent upon the GAG class], and polymeric chain extension from the non-reducing end.[§] With the exception of HA which is unmodified, the resultant polymer is extensively modified through uronic acid [GlcA to iduronic acid (IdoA)] epimerisation (heparin, HS, DS), *N*-deacetylation coupled with *N*-sulfation (heparin, HS), and *O*-sulfation (all GAGs) creating heterogeneous structures (Figure 1).[15]

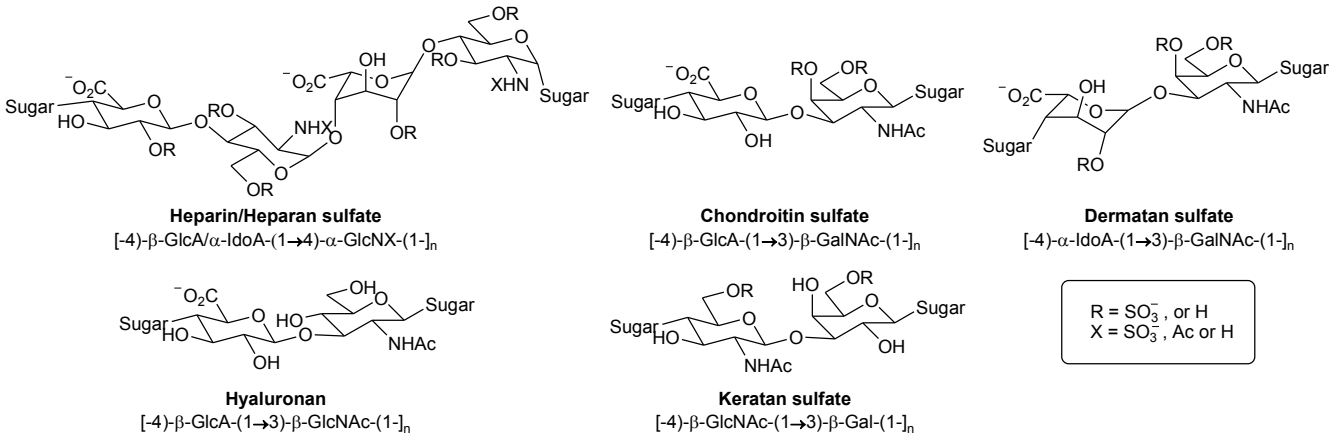


Figure 1. GAG-polysaccharide classes. [§]

Functionalisation through the attachment of fluorescent labels, spin labels, or biotinylation of these linear oligosaccharides might be envisaged at either the reducing or non-reducing end (or both) to allow the wealth of modern biophysical techniques to be applied to the determination of the GAG oligosaccharide solution conformation, and to obtaining vital information about protein-GAG interactions. The method by which the GAG oligosaccharide sample under study has been prepared (Section 2) determines which is the most appropriate route to functionalisation (Section 3). But notwithstanding which method is used, the production of functionalised GAG oligosaccharides poses a considerable challenge due to the polyanionic character and structural diversity which they display along the GAG chain. The applications of these strategies highlighted in Section 4 are thus notable achievements in the field.

2. Sample preparation

X-ray crystal structures, a limited number of NMR studies and molecular modelling of some GAG-protein complexes suggest that the most common length within the GAG polymer for protein recognition and binding consists of four to six sugar units.[6,16] However, these studies have invariably

used highly sulphated heparin-derived oligosaccharides which may mask the true specificity of complex formation with less sulfated species *in vivo*. Although some evidence suggests that charge density is a major component of recognition for some proteins,[5] in others evidence points to a significant role for the relative placement of key recognition motifs within the GAG sequence.[17]

As a further complication, the strength of binding does not always correlate with the biological activity; and in some instances less sulfated species have been shown to be more active.[18] If this seemingly contradictory body of data is to be reconciled, sample preparation and isolation methods must address sample homogeneity with respect to the length of the sugar chain as well as its detailed saccharidic make up. Pure, well characterised species of an appropriate degree of polymerisation (dp) and defined sulfation pattern are urgently needed.

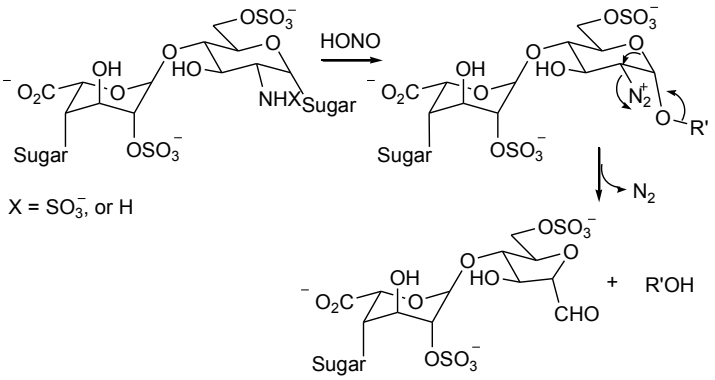
Two principal methods have been used to achieve the depolymerisation of native GAG-polysaccharides, i.e. chemical and enzymatic cleavage (Section 2.1).[19] Both of these methods have found application in the commercial preparation of low molecular weight heparins for clinical anticoagulative treatments.[20] For structural and biological studies these methods may be used to supply microgram to milligram quantities of individual dp fractions of a GAG oligosaccharide which may then be further purified into their individual structural components (Section 2.1.3). Alternatively, sample homogeneity may be achieved by the total chemical or chemoenzymatic synthesis of the GAG oligosaccharide (Section 2.2), or by global chemical or enzymatic processing (e.g. selective desulfation) of the GAG chain (Section 2.3).

2.1 Preparation of GAG oligosaccharides through depolymerisation

2.1.1 Chemical cleavage

The chemical depolymerisation of GAGs is frequently achieved either using nitrous acid, or by radical cleavage in aqueous peroxide.[21] Cleavage of 2-amino-2-deoxy-D-glucosidic bonds by nitrous acid is initiated by nitrosation of the amino group of the sugar,[‡] followed by loss of nitrogen with a ring contraction of the D-glucosamine residue to the 2,5-anhydro-D-mannose coupled to elimination of the aglycone.[22] The 2,5-anhydro-D-mannose is thus the new reducing terminal of the oligosaccharide formed in this deamination reaction (Scheme 1). The terminal aldehyde functionality which results is ideally suited to functionalisation by reductive amination, or hydrazone formation (as discussed in Section 3.1), and typically shows enhanced reactivity relative to comparable reactions of an aldohexose.[19] Short reaction times (typically 10 min) mean that little if any desulfation occurs along the GAG chain. However, the conformational changes introduced by the artificial terminal residue are

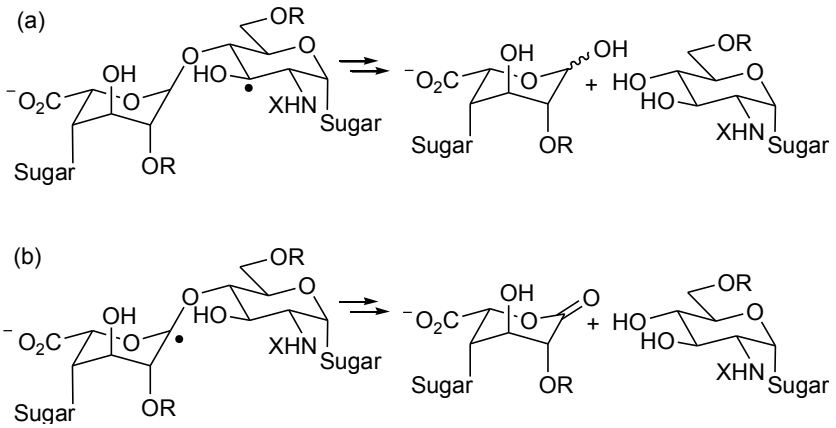
likely to impact upon the solution conformation and protein-binding of any GAG oligosaccharide probe generated in this manner.



Scheme 1. Nitrous acid induced depolymerisation.

The other method chiefly employed in the chemical preparation of short-chain GAGs is radical depolymerisation, using hydrogen peroxide in the presence of a transition metal catalyst such as copper(II) or iron(II).[23] Typical reaction conditions include the use of $Cu(OAc)_2/H_2O_2$ at pH 7.5, Fenton's reagent $FeSO_4/H_2O_2$ at pH 4.5, and $Cu(OAc)_2/H_2O_2$ at pH 4.1.[23-25] Depolymerisation of CS and DS with H_2O_2 in the presence of a copper(II) catalyst has been shown to result in structural changes which render the depolymerised material more resistant to enzymatic degradation.[26] Copper(II)-catalysed depolymerisation of heparin is generally more selective than iron(II)-catalysed depolymerisation and tends to occur adjacent to 2-*O*-sulfated IdoA residues; it is thought that the selectivity for depolymerisation is conferred by binding of the copper(II) ion to the GAG-polysaccharide, and this is consistent with a reduced selectivity for attack induced by the more weakly binding iron(II) ion.[23] Recent studies have shown that the SO_3^-/CO_2^- ratio (an index of the total sulfate group content) of "low molecular weight" heparin obtained under copper(II)-catalysed depolymerisation conditions is slightly higher than that of the parent heparin, whilst the *N*-sulfate content is hardly changed at all.[25]

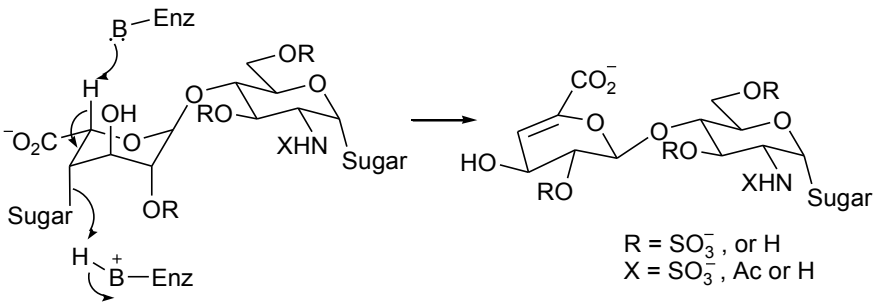
Two sites for the initiation of GAG depolymerisation have been proposed: radical abstraction at C2 or C3 of either a uronic or glucosamine residue; or alternatively radical abstraction at the anomeric position (Scheme 2).[27] A stable nitroxide radical, resulting from hydroxyl radical attack on *N*-sulfated or free amino groups, has also been observed under these conditions.[28] Overall radical depolymerisation is not well suited to generating homogeneous GAG oligosaccharide samples because it provides both odd- and even-numbered oligomers and results in many minor functional group alterations especially at the reducing end.[27]



Scheme 2. Proposed radical formation and resultant cleavage products: (a) C3 (or C2) abstraction followed by β-scission; (b) anomeric abstraction followed by oxidation and glycosidic bond cleavage.

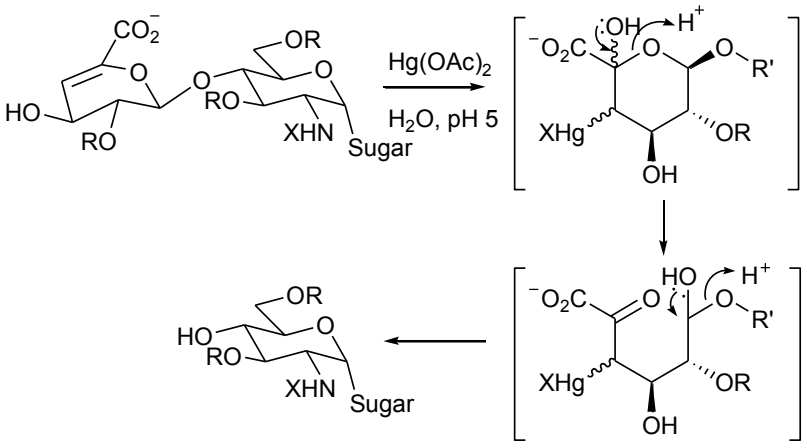
2.1.2 Enzymatic cleavage

Enzymatic digestion of GAGs by bacterial or fungal lyases is a well-established technique for their depolymerisation. These eliminase enzymes fall into three classes depending on their primary substrate (chondroitinase, heparinase, and hyaluronidase);[29] which are further subdivided according to their sequence specificities (chondroitinase AC, B, C and ABC;[30] and heparinase I, II and III[31]). Mechanistically the lyase enzyme brings about the depolymerisation of the GAG backbone through a β-eliminative cleavage as shown in Scheme 3.[29] Lyases are potentially able to degrade GAGs down to purely disaccharides. In reality, substrate complexity usually results in a preponderance of di- and tetra-saccharides (dp2-dp4),[32] mixed with larger resistant sequences, dependent on the individual GAG substrate used. Controlled partial digestions can be used to generate more complex mixtures. After separation by size-exclusion chromatography (SEC), these give a range of individual size fractions, typically from dp2 to ≥dp12.[33]



Scheme 3. Proposed lyase cleavage mechanism.

The unsaturated uronic acid (ΔUA) at the non-reducing terminus, which results from lyase cleavage, has a distinctive chromophore at 230 nm (ε 5000–6000 M^{-1} cm^{-1}) that may be used to monitor the progress of the reaction.[34] It also provides a unique functional group handle that may prove useful for further chemical functionalisation of the GAG oligosaccharide. Both IdoA and GlcA give the same ΔUA upon cleavage, thus important sequence information may be lost. This ΔUA residue can exist in either the $^2H_1$ or $^1H_2$ conformation; the equilibrium between these two conformers is controlled by its sulfation pattern.[35] NMR studies show that $^1H_2$ is generally the favoured conformation,[36] although both forms appeared in the same unit cell in a crystal structure,[37] which indicates that they have nearly the same energy. Where this modified uronic acid is not required at the non-reducing terminus it is readily removed in the presence of mercuric salts e.g. $HgCl_2$ or $Hg(OAc)_2$,[38] through an oxymercuration reaction which is rapidly followed by hydrolysis of the resultant hemi-ketal (Scheme 4).



Scheme 4. Oxymercuration and loss of the terminal $\Delta^{4,5}$ unsaturated uronic acid residue of lyase-cleaved GAG oligosaccharides.

2.1.3 Separation of GAG oligosaccharides

The complex mixture of oligosaccharide obtained via chemical or enzymatic cleavage requires separation by SEC to give GAG oligosaccharides fractions of known dp.[39] When lyase-generated these are readily detected by monitoring the absorption at 230 nm (Figure 2), whilst chemically depolymerisaed GAG oligosaccharides might require the attachment of a functional tag (e.g. fluorophore) to aid this process (though carbonyl bond absorption at 206 nm is possible if running in a low-absorbance background).

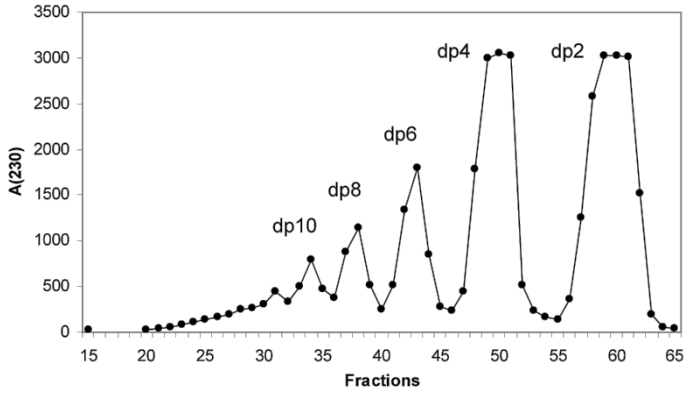


Figure 2. Size separation of heparin oligosaccharides obtained by heparinase I digestion of bovine heparin on a Biogel P10 size exclusion column.

Separation of individual components from particular size fractions of any GAG oligosaccharide must then be achieved. The structural similarities of individual components (which may vary only in sulfation pattern, or uronic acid configuration) combined with the highly anionic nature of the GAG oligosaccharides means that further separation of individual components is most frequently achieved using strong anion-exchange HPLC (SAX-HPLC, Figure 3);[40] ion-pair reverse phase HPLC (IP-RP-HPLC);[41,42] capillary electrophoresis (CE);[43] or polyacrylamide gel electrophoresis (PAGE).[44]

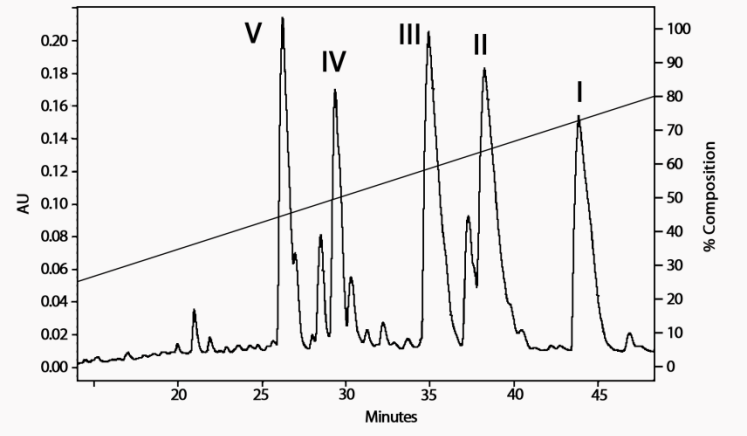


Figure 3. Semi preparative SAX-HPLC of the dp4 fraction of enzymatically digested bovine heparin eluted using a 60 min linear gradient from 0 to 1 M sodium chloride (pH 3.5). Peaks **I-V** represent the following species: **I**: α-ΔUA(2S)-(1→4)-α-GlcNS(6S)-(1→4)-α-IdoA(2S)-(1→4)-GlcNS(6S); **II**: α-ΔUA(2S)-(1→4)-α-GlcNS(6S)-(1→4)-β-GlcA-(1→4)-GlcNS(6S); **III**: α-ΔUA(2S)-(1→4)-α-GlcNS(6S)-(1→4)-α-IdoA(2S)-(1→4)-GlcNS; **IV**: α-ΔUA(2S)-(1→4)-α-GlcNS-(1→4)-β-GlcA-(1→4)-GlcNS(6S); **V**: α-ΔUA(2S)-(1→4)-α-GlcNS-(1→4)-α-IdoA(2S)-(1→4)-GlcNS.

The individual saccharide composition and sequence of any particular fraction may then be determined by disaccharide analysis,[41,45] HPLC- or PAGE- based sequencing, or by using mass spectrometric-,[46] or NMR-based,[47] methods.

2.2 Total chemical or chemoenzymatic synthesis of GAG oligosaccharides

Recent advances in oligosaccharide synthesis[48] have rendered the preparation of native GAG oligosaccharides (rather than simplified GAG-mimetics) through total chemical synthesis more attractive. Total synthesis offers the potential for the generation of GAG oligosaccharides with defined sulfation patterns,[49] on a larger scale than is readily achieved through the controlled degradation of native material. The synthetic anticoagulant, antithrombin-binding pentasaccharide, fondaparinux,[50] based upon the native heparin sequence, has recently been marketed worldwide under the trade name Arixtra® (Figure 3). Early synthetic endeavours have been reviewed elsewhere,[51] but notable recent achievements in this area include: an efficient one-pot strategy for the synthesis of heparin and HS oligosaccharides, in which the formation of uronic acids is achieved by selective oxidation of the C-6 hydroxyl group after oligosaccharide synthesis;[52] the synthesis of sulfated heparin-like oligosaccharides on a soluble polymer support;[53] the synthesis of a HS tetrasaccharide with both free and *N*-acylated amino groups;[54] the assembly of well-defined CS oligosaccharides using convergent synthetic approaches;[55,56] and the synthesis of microarrays of synthetic heparin oligosaccharides of chain length dp2-dp6, using automated solid phase synthesis.[57]

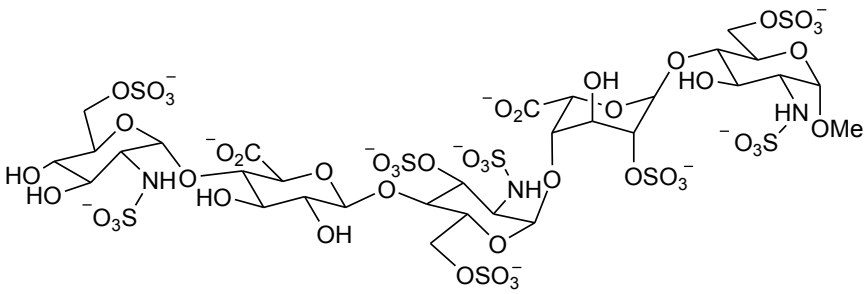
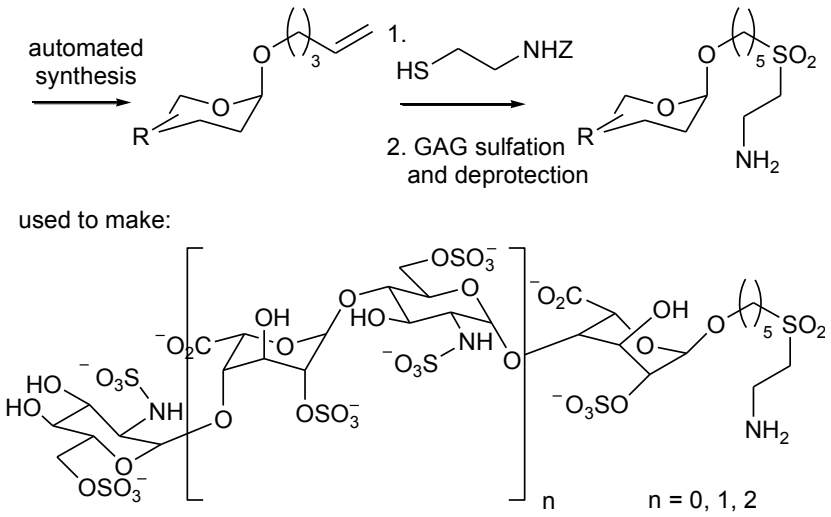


Figure 4. Arixtra®, a synthetic antithrombin-binding pentasaccharide.

One of the principal advantages of a total chemical synthetic approach is that a number of activating/reactive groups may be readily introduced to the developing oligosaccharide to allow site-specific attachment to functional tags. This is exemplified by the terminal pentenyl glycoside which is generated on release of an oligosaccharide from the solid support in Seeberger's

automated synthesis protocol. This is readily converted to an amine-functionalised GAG oligosaccharide (Scheme 5) appropriate for immobilisation on amine-reactive CodeLink slides.[58] Microarrays displaying a small library of synthetic heparin oligosaccharides generated using this technology have recently been used to profile heparin-chemokine interactions.[59]

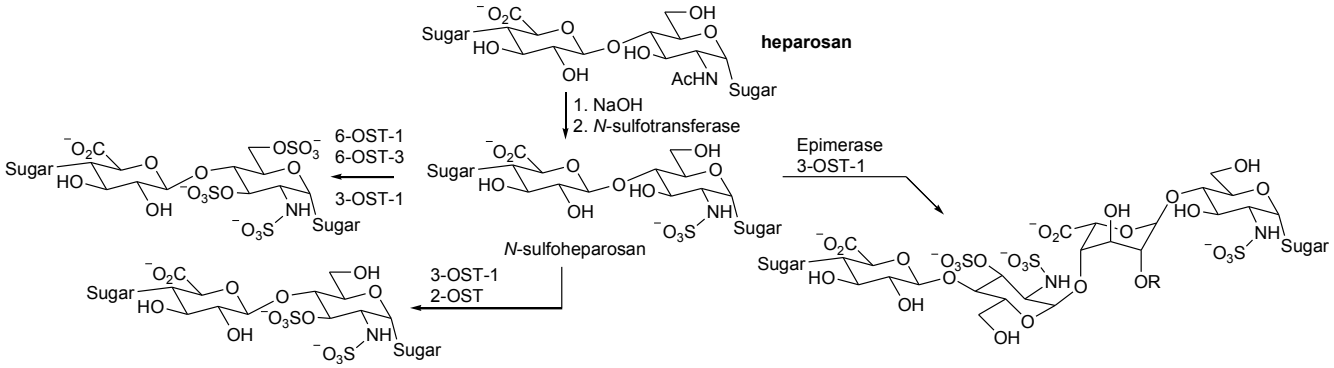


Scheme 5. Pentenyl glycoside conversion to an amine-functionalised GAG oligosaccharide.

2.3 Chemical and enzymatic modification of GAG precursors

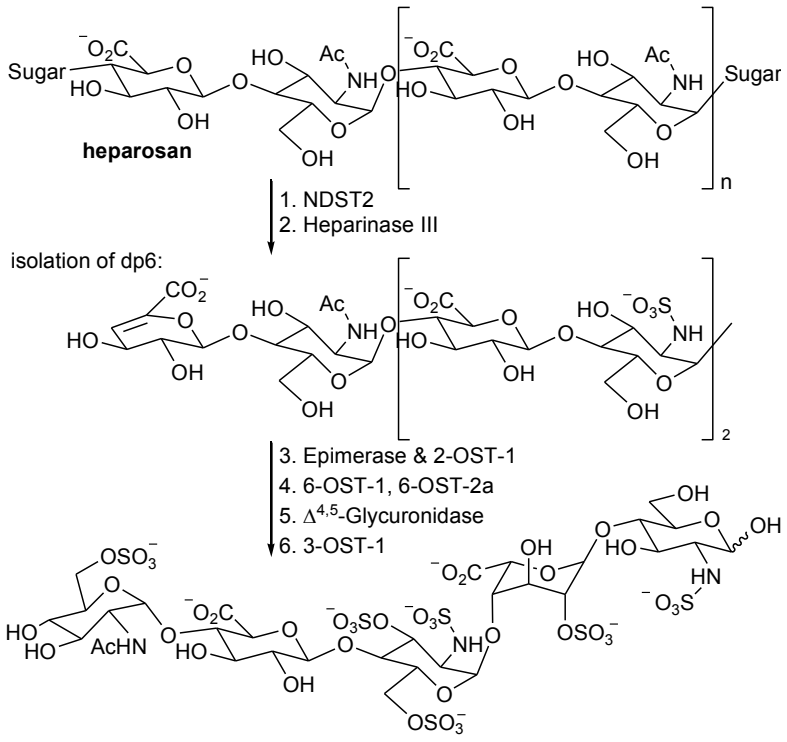
Despite notable successes in chemical and chemoenzymatic synthesis, longer chain length GAG oligosaccharides (>dp6) are still largely inaccessible using current oligosaccharide synthetic methodology; hence *in vitro* systems using biosynthetic enzymes offer a promising alternative approach.[60] Particularly promising is the prospect of using sulfotransferase enzymes for highly selective sulfate transfer onto oligosaccharide or polysaccharide precursors.[15,61] Sulfotransferases allow access to sulfate patterns that are difficult to achieve using standard chemical methods, and the different isoforms of each transferase (e.g. 6-OST-1 and 6-OST-2a) confer exquisite substrate selectivity. This approach is exemplified by the work of Liu et al.; starting from heparosan, a capsular polysaccharide of the *E. coli* K5 strain which is the non-sulfated and unepimerised equivalent of heparin/HS, an enzyme-based combinatorial approach to the

synthesis of a polysaccharide library with different sulfation patterns was developed (Scheme 6).[62] The principal drawback of this approach for biophysical, and more detailed biological structure-activity studies is that it has not yet been developed to a position where the constitutional homogeneity of the resultant synthetic polysaccharides can be guaranteed.



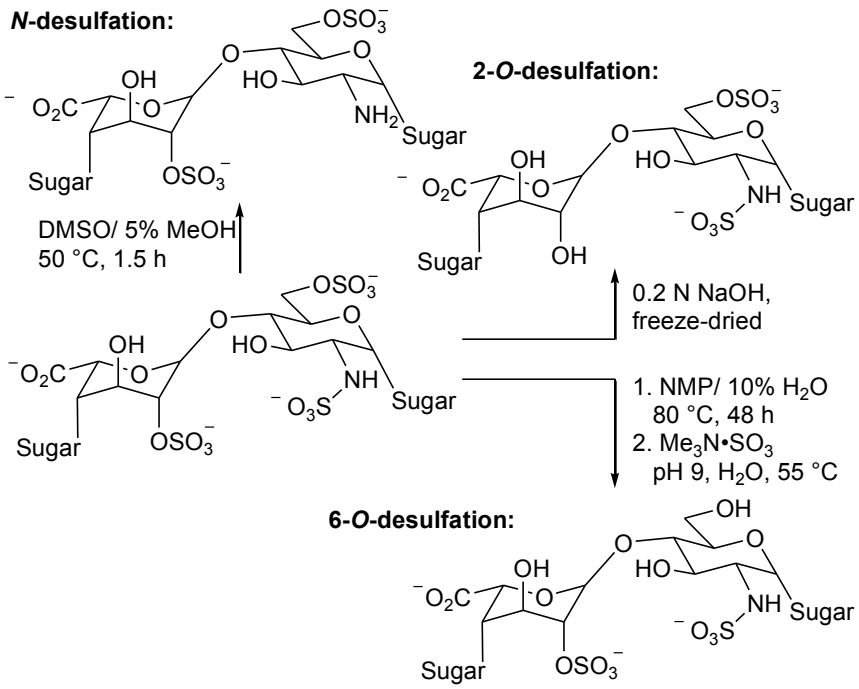
Scheme 6. A combinatorial enzymatic approach to the synthesis of GAGs from heparosan.

In another outstanding example of the use of an enzyme-based modification approach to functional GAG oligosaccharide synthesis, the antithrombin-binding pentasaccharide of heparin/HS was synthesised in just 6 steps from heparosan (Scheme 7).[63] This was achieved using the enzymes *N*-deacetylase-*N*-sulfotransferase (which effects the dual transformation of the hexosamine residues from GlcNAc to GlcNS); heparinase III (chain degradation followed by isolation of the dp6 fraction); epimerase and 2-OST-1 (GlcA to IdoA interconversion and 2-*O*-sulfation of IdoA); 6-OST-1 and 6-OST-2a (glucosamine-6-*O*-sulfate formation); $\Delta^{4,5}$-glycuronidase (to remove the non-reducing terminal ΔUA and generate the pentasaccharide); and 3-OST-1 (to effect the final 3-*O*-glucosamine sulfation).



Scheme 7. Antithrombin-binding pentasaccharide synthesis by enzymatic modification of heparosan.

Chemical processing of the sulfated backbone of GAGs may be achieved in a number of ways,[64] but has been most comprehensively studied for heparin. *N*-desulfation of the pyridinium salt of heparin is highly efficient,[65] and is usually followed by *N*-acetylation to protect the highly reactive amino groups which result.[66] A straightforward 2-*O*-desulfation protocol for heparin described by Ishihara et al. is also highly efficient and selective.[67] The 6-*O*-desulfation of the glucosamine residues, leaving the 2-*O*-sulfation of IdoA intact is the least selective process.[68] Under all conditions studied this reaction is accompanied by partial loss of the highly labile sulfamido group; however, this may be readily reversed through specific *N*-resulfation (Scheme 8).[69,70]



Scheme 8. Specific desulfation reactions of heparin.

3. Chemical Functionalisation

General methods for the introduction of structural motifs along the oligosaccharide chain of heparin structures (e.g. *N*- and *O*-acylation, sulfate substitution, carboxylate ester and amide formation) have been comprehensively reviewed.[71] This section therefore focuses more specifically on methodology appropriate for the selective introduction of functional labels at either the reducing end (Section 3.1) or non-reducing end (Section 3.2) of the GAG oligosaccharide, and how these methods are most appropriately combined with the different

procedures for GAG oligosaccharide sample preparation.

3.1 Reducing end functionalisation

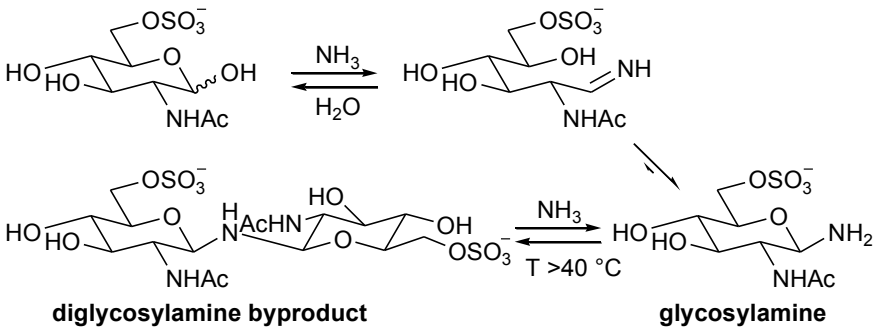
Most GAG oligosaccharide structures possess a reducing terminus with a uniquely reactive anomeric centre which may be modified in a number of different ways in common with other carbohydrates.[72] (GAG oligosaccharides produced by chemical cleavage employing nitrous acid have a modified reducing terminus, as discussed in Section 2.1.1, which is also highly reactive.) For this reason reducing end functionalisation has been widely exploited, for example for the attachment of fluorophores to GAG oligosaccharides to aid disaccharide analysis,[41,45] and purification,[73] and to enable binding affinity studies by gel mobility shift assays.[74] However, not all methods developed for oligosaccharide functionalisation at the reducing end are applicable to GAG oligosaccharides and care must be taken to ensure that reaction conditions do not give rise to undue structural modifications of the GAG oligosaccharide under study.[75,76] Thus for example, it has been shown that under mildly basic conditions a terminal GlcNS(6S) residue of GAG oligosaccharides may be transformed through C2-epimerisation into the corresponding ManNS(6S).[75] In addition the polyanionic nature of GAG oligosaccharides limits options for their functionalisation, as sodium salts of heparin are only soluble in water and aqueous buffer systems, or water combined with aqueous-miscible solvents such as formamide. Pyridinium or ammonium salt forms of GAG oligosaccharides have been shown to have a greater range of solubility, and reactions have been demonstrated in DMF, DMSO and even CH_2Cl_2.[71]

3.1.1 Glycosylamine formation and amine derivatisation

Kochetkov amination,[77] i.e. the formation of a glycosylamine from a glycoside through treatment with an ammonia source, may be achieved under a number of different reaction conditions in complex oligosaccharides.[78] This versatile method has underpinned the preparation of numerous neoglycoconjugates since the resultant terminal glycosylamine provides a convenient site for chemoselective conjugation and modification.[72] Typical reaction conditions for the Kochetkov reaction involve mild heating (~40 °C) of the oligosaccharide with an ammonia source [e.g. NH_3(aq.)/NH_4HCO_3,[79] $NH_4CO_2NH_2$,[80] NH_4HCO_3,[81] or $(NH_4)_2CO_3$[82]] for an extended reaction period (>16 h). The use of organic solvents such as methanol, or DMSO can lead to higher yields of the unstable glycosylamine products. However, the use of a large excess of the ammonia source (5-20 equivalents) means that this reaction is mostly confined to comparatively small sample sizes (10-100 mg), and volatile ammonium salts are preferred since they may be removed at the end of the reaction by lyophilisation, or by heating in methanol (~70 °C). Nonetheless, recent advances such as the application of microwave technology to reduce reaction times to ~90

min, suggest that there is still considerable potential for application of the Kochetkov reaction in glycobiology.[83,84]

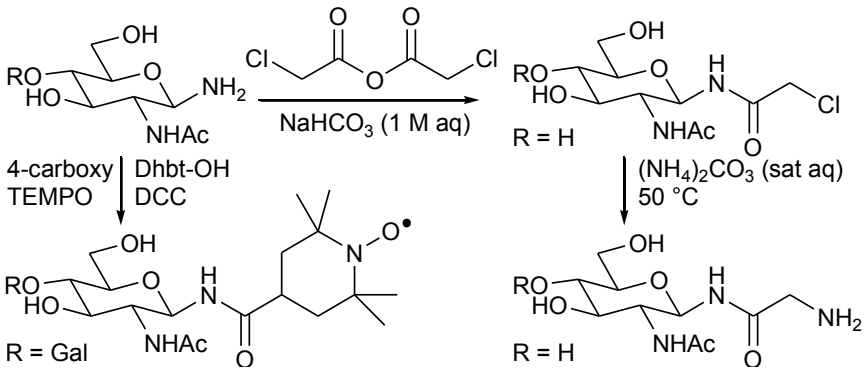
Since the glycosylamine is predominantly isolated as its ring-closed β-anomer, rather than the corresponding open chain imine, the structural information associated with this ring is preserved rendering this an attractive method for the functionalisation of GAG oligosaccharides (Scheme 9).[81] Typically, the only side-product formed in a Kochetkov amination is the diglycosylamine;[82] production of which is minimised at lower reaction temperatures (<40 °C).[84] (It is noteworthy that a dilute solution of ammonium bicarbonate is frequently used as a carrier for GAG oligosaccharides, demonstrating that they are stable to these conditions for short contact periods.)[85] However, there is as yet only one report of the successful application of the Kochetkov reaction to sulfated monosaccharides such as those found in GAG oligosaccharides.[82]



Scheme 9. Kochetkov amination of GlcNAc(6S).

Whilst glycosylamines can be relatively easily obtained they are very labile in slightly acidic or neutral aqueous media; thus once formed they are typically acylated to confer additional stability.[81] The reaction of purified complex oligosaccharides with ammonium bicarbonate followed by acylation with fluorophores such as dansyl chloride or carboxyfluorescein has been shown to be an effective means of derivatisation, although reaction yields and anomeric ratios have been shown to be somewhat dependent on the steric bulk of the acylating agent.[81] However, reaction with chloroacetic anhydride followed by ammonolysis of the resultant chloroacetamido group (Scheme 10), has been shown to give the β-anomeric *N*-glycine derivatives with high selectivity.[81] More recently, coupling of glycosylamines with FmocAsp(OH)-O^{t}Bu in the presence of HOBt/HBTU, and subsequent acidic deprotection has been shown to give the corresponding Fmoc-protected glycosylamino acids,[86] and the preparation of an activated ester intermediate of the spin label 4-carboxy-TEMPO through treatment with HODhbt/DCC has allowed reaction with a LacNAc-derived glycosylamine.[87] Stabilisation has also been achieved

through reaction of the glycosylamine with 2-iminothiolane hydrochloride to generate the corresponding *N*-imino glycosylamidine.[83]



Scheme 10. *N*-acylation of glycosylamines.

Direct formation of functionalised glycosylamines is an alternative procedure to a Kochetkov amination / acylation type sequence.[88] Glycosylamines prepared using this strategy include those of 4-aminobenzoic butyl ester (4-ABBE), 4-aminobenzoic acid (4-ABA), and 2-aminopyridine (2-AP) as shown in Figure 5.[88,89] Glycosylamines formed by reaction with 4-ABA have been analysed by CE–ESI-MS–MS, where it has been shown that the closed ring structure of the glycosyl amine provides more information on the linkage and anomeric conformation than their secondary amine counterparts formed by reductive amination (Section 3.1.3).[89] With nitrous acid depolymerised GAG oligosaccharides the exocyclic aldehyde forms a stable Schiff's base with an amine.[90]

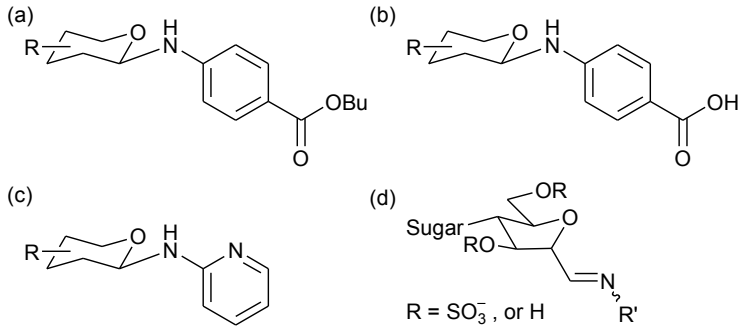
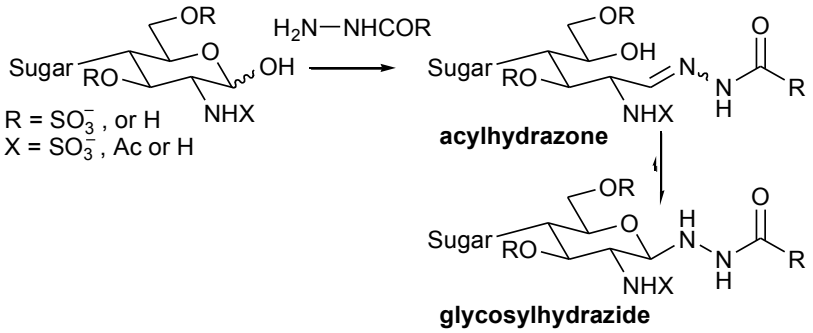


Figure 5. (a-c) Glycosylamines formed by the direct reaction of oligosaccharides with 4-ABBE, 4-ABA, and 2-AP respectively. (d) Schiff's base formed by coupling nitrous acid depolymerised GAGs with amines.

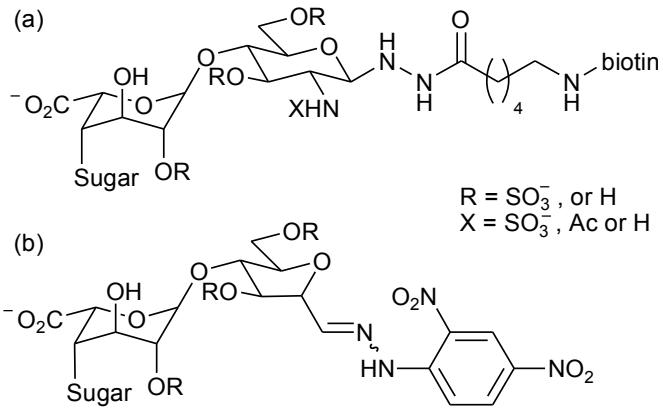
3.1.2 Glycosylhydrazide formation

Treatment of GAGs with hydrazine (H_2NNH_2) is routinely used as a means of removing any *N*-acyl groups in the polymer chain.[91,92] However coupling of the reducing sugar terminus of GAG oligosaccharides with a hydrazide ($H_2NNHCOR$) affords an acylhydrazone. Hydrazides are attractive for coupling reactions with GAGs as they retain their nucleophilicity in acidic aqueous media, and the acylhydrazone which is formed is comparatively stable. Equilibration to the tautomeric ring-closed glycosylhydrazide is generally favoured, and this exists predominantly as its β-anomer (typically >90:10 β:α, Scheme 11).[93,94] The acylhydrazone typically has an H-1 signal in the range 7.5-7.6 ppm in D_2O, whereas the β-glycosylhydrazide has an H-1 signal of 4.2-4.3 ppm.[93] The acylhydrazide group may also show restricted bond rotation about the N-acyl bond, resulting in complex NMR spectra for the products of these reactions.[93]



Scheme 11. Glycosylhydrazide formation.

Complex oligosaccharides,[95] including heparin-derived oligosaccharides,[96] have been attached directly to hydrazide-coated glass slides to form microarrays through a β-glycosylhydrazide linkage. The reagent 6-(biotinyl)-aminocaproyl hydrazide (BACH) has also been shown to react directly with commercial low molecular weight heparins (Figure 6a).[97] Glycosylhydrazide formation is an attractive approach to functionalisation as it is chemoselective, does not require the use of coupling reagents, and the native pyranose form of the reducing sugar is retained.

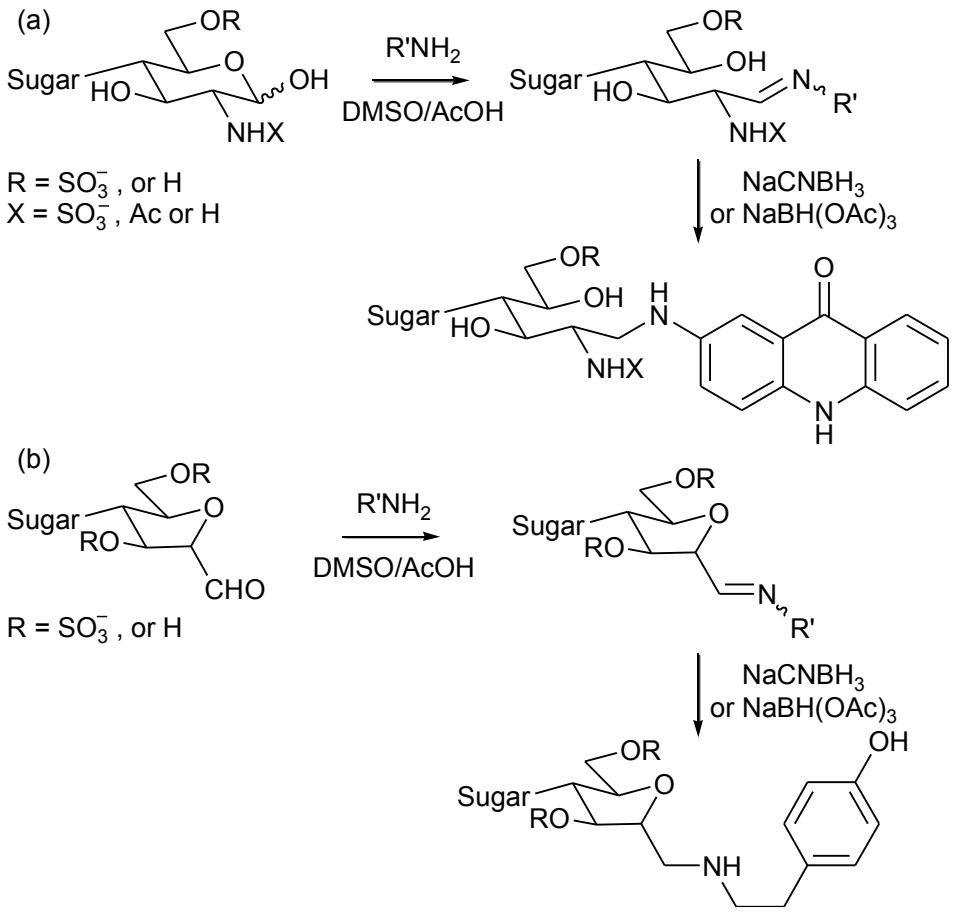


Where a functionalised hydrazine is reacted with GAG oligosaccharides terminated by an anhydromannose resulting from deaminative cleavage, formation of the glycosylhydrazone is favoured.[98] It has been shown that reaction of intracellular HS degradation products with 2,4-dintrophenylhydrazine (DNP) forms a hydrazone which may be visualised through immunofluorescence using anti-DNP (Figure 6b).[99]

3.1.3 Reductive amination

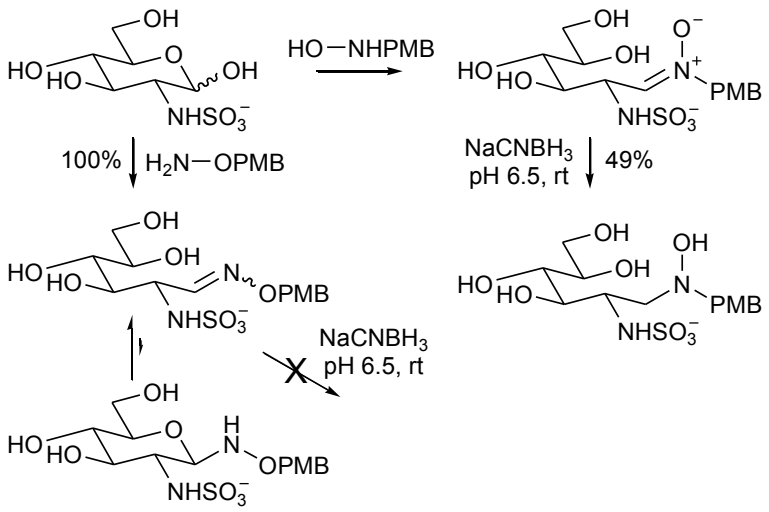
A widely-used method for the functionalisation of the reducing end of oligosaccharide chains is reductive amination with an appropriate amine to give the corresponding secondary amine.[72-74,100] The reductive amination of GAG oligosaccharides typically requires extended reaction times at pH 4-5 in the presence of an excess of amine to form the initial imine, which is reduced *in situ* with an excess of sodium cyanoborohydride ($NaCNBH_3$) or sodium triacetoxy-borohydride [$NaBH(OAc)_3$]. Where this reaction is carried out on GAG oligosaccharide samples resulting from enzymatic or radical depolymerisation, the reductive amination procedure results in the linear form of the reducing terminus sugar, with the associated loss of any structural information incorporated in this sugar residue (Scheme 12a). These reactions are also applicable to GAG oligosaccharides prepared through treatment with nitrous acid, where the five-membered ring formed during the cleavage reaction is retained (Scheme 12b).[101] Although it requires relatively harsh conditions, the reductive amination of GAG oligosaccharides has been used for the introduction of fluorophores to aid separation[73,102] GAG-sequencing,[103] studies of protein-GAG interactions,[74] and mass spectrometric analysis,[46] and for the i oligosaccharides on a range of surfaces.[104]

← **Figure 6** (a) Glycosylhydrazide formation with lyase-cleaved heparin using BACH; and (b) glycosylhydrazone formation with *in vivo* radical depolymerised GAGs using DNP.



Scheme 12. Reductive amination: (a) of lyase or radical depolymerised GAGs with 2-aminoacridone (AMAC); (b) of nitrous acid depolymerised GAGs with tyramine.

Despite the enhanced nucleophilicity in acidic aqueous media of hydrazides over their amine counterparts, the reductive amination of GAG-acylhydrazones is not often used as a means of GAG functionalisation, reflecting the comparative stability of the acylhydrazones themselves. However, another variant of the reductive amination procedure, reductive oxyamination, has been proposed as an alternative. The reaction of *N*-substituted hydroxylamines and $NaCNBH_3$ to give *N*,*N*-disubstituted hydroxylamines proceeds selectively and in quantitative yield under mild conditions (aqueous solution, pH 6.5, rt) for a range of monosaccharides.[105] Unfortunately GlcNS, which was used as a model for the reducing terminus of enzyme-cleaved GAG oligosaccharides, gave a greatly reduced yield (Scheme 13).[105] Reaction with the isomeric *O*-substituted hydroxylamines results in the formation of *E/Z* oxime mixtures and proceeds in quantitative yield for GlcNS.[105] In contrast to acylhydrazones these oximes are thought to exist predominantly in the ring-opened form, and they are not amenable to reductive amination.[105] Oxime derivatives themselves have been exploited though, and the disaccharide ΔUA(2S)-GlcNS(6S) has been immobilised through an oxime linkage in a recent oligosaccharide microarray.[106]



Scheme 13. Reaction of GlcNS with *N*- and *O*-substituted hydroxylamines.

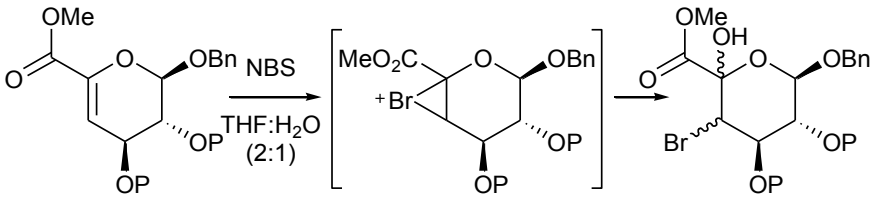
3.2 Non-reducing end functionalisation

To prepare double-labelled substrates for use in structural studies (e.g. determination of changes in solution conformation of GAG oligosaccharides with varying sulfation using TR-FRET),[107] selective labelling at the non-reducing end is also required. Bacterial lyase-catalysed cleavage of GAGs produces oligosaccharides containing a ΔUA at the non-reducing terminus.[29] This unique feature in the heavily sulfated GAG chain makes an attractive target for selective functionalisation. However, only a limited number of approaches to functionalisation of this reactive moiety have been reported, as discussed below.

3.2.1 Double bond functionalisation

Functionalisation of the captodative $\Delta^{4,5}$ double bond of lyase-cleaved GAG oligosaccharides is challenging as it exhibits both acrylic acid and enol ether electronic character.[108] Oxymercuration, of the $\Delta^{4,5}$ double bond in these unsaturated uronic acids by mercury salts in H_2O and the rapid glycosidic bond cleavage which ensues, has been used extensively for the selective removal of the terminal unsaturated saccharide moiety in lyase-cleaved GAGs since its introduction in 1987 (Scheme 4).[38] Surprisingly in this context, trapping of a mercurinium intermediate formed under similar conditions [$Hg(OAc)_2$, THF/H_2O, 40 °C, 2 h] by thiol modified surfaces has recently been reported.[109] Oxidative functionalisation of the double bond of unsaturated uronic acids through dihydroxylation is similarly known to result in cleavage of the non-reducing terminal sugar.[110] Notwithstanding this facile degradation pathway, the reaction of variously protected ΔUA's with

N-bromosuccinimide (NBS) in aqueous THF to generate the corresponding bromohydrins (Scheme 14) as a mixture of stereoisomers in good yields has been reported.[111] But perhaps not surprisingly, direct application of this methodology to the double bond of lyase-cleaved heparin-derived GAG-disaccharides results in extensive decomposition, most probably through glycosidic bond cleavage.[112]

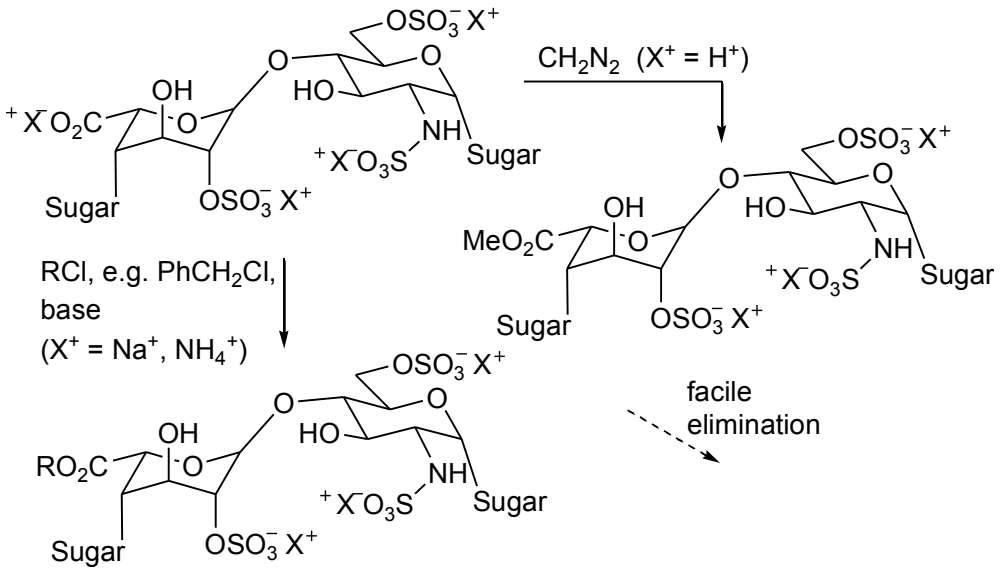


Scheme 14. Functionalisation of the captodative double bond in a ΔUA.

Other possible means of chemical functionalisation of GAG ΔUA double bonds, such as Michael addition to the captodative double bond,[113] or Heck reaction of this acrylate-like functionality,[114] do not appear to have been attempted on these challenging targets.

3.2.2 Carboxylic acid functionalisation

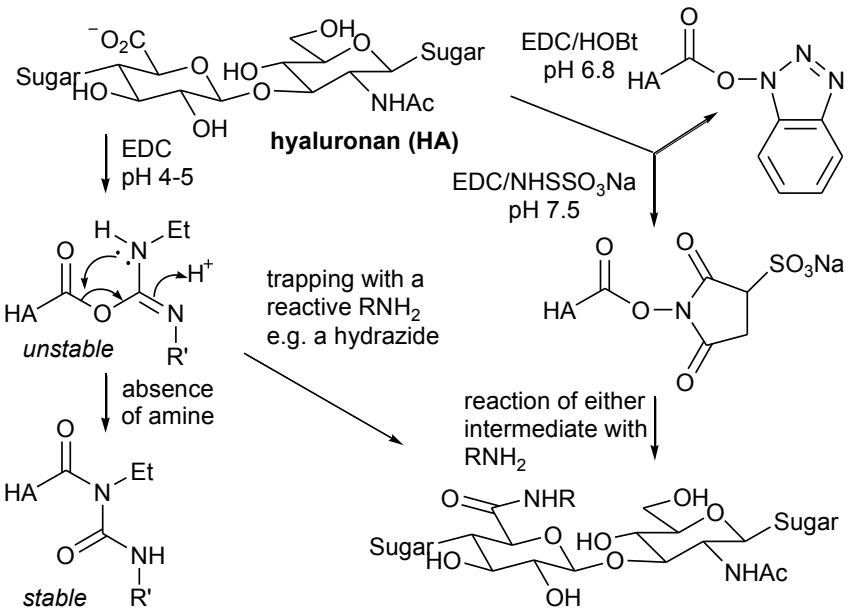
Reaction of the carboxylic acid functionality to give either an ester or amide provides an attractive alternative to double bond functionalisation as GAGs and GAG oligosaccharides are compatible with a range of standard coupling reagents/ conditions, and the resultant modified GAGs are relatively stable.[71] The free acid form of heparin reacts rapidly with diazomethane to give the uronic acid methyl ester,[115] whilst the sodium or ammonium salt reacts with an alkyl halide (e.g. benzyl chloride) in the presence of a base to give the corresponding ester (Scheme 15).[116] Although uronic esters are stable by comparison with the products of double bond functionalisation discussed in Section 3.2.1, esterification lowers the pK_a of the H-5 of the uronic acid, facilitating proton abstraction and subsequent elimination under basic conditions.[71] As yet the selective esterification of the carboxylic acid at the non-reducing terminus, for instance through using a bulky alkylating agent, has not been reported.



Scheme 15. Ester formation in GAGs.

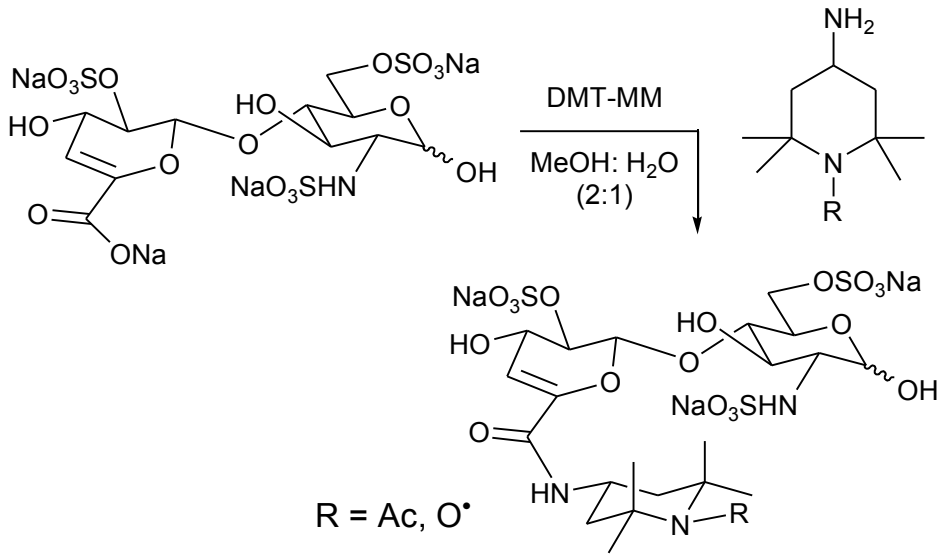
The range of coupling reagents available for amide bond formation has increased dramatically in recent years, and many new water-soluble coupling reagents have been developed opening up new opportunities for peptide bond formation.[117] Of these coupling agents, the water soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC or EDAC) has been shown to couple a range of hydrazides to the uronic acids of HA, CS and heparin at pH 5.2,[118] and also to cross-link HA through its uronic acids via reaction with dendritic hydrazides.[119] *O*-substituted hydroxylamines have also been coupled to the carboxylate groups of HA with EDC at pH 4.75.[120]

In contrast, it has been reported that the reaction of the carboxylates of HA with primary amines at pH 4.5 gives rise to the *O*-acyl urea of EDC (which rapidly rearranges under acidic conditions to the more stable *N*-acyl urea), with none of the expected amide coupled product.[121,122] Successful coupling to amines requires the use of HOBt in conjunction with EDC at pH 6.8, or conversion to an intermediate *N*-hydroxysulfosuccinimide ester at pH 7.5 (Scheme 16).[123,124] Formation of the *N*-acyl urea has subsequently been used as the basis of hydrogel formulation strategies.[124] EDC has been reported to couple eight different GAG-disaccharides with the fluorophore 7-aminonaphthalene-1,3-disulfonic acid (ANDSA);[125] the derivatised disaccharides were then analysed by capillary electrophoresis.



Scheme 16. Peptide and *N*-acyl urea formation reactions of hyalurronan.

The water soluble coupling agent 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM)[126,127] has also been employed in amide bond formation to GAG oligosaccharides, allowing the direct coupling of 4-amino-TEMPO to a heparin-derived GAG-disaccharide with good conversion (Scheme 17).[128]



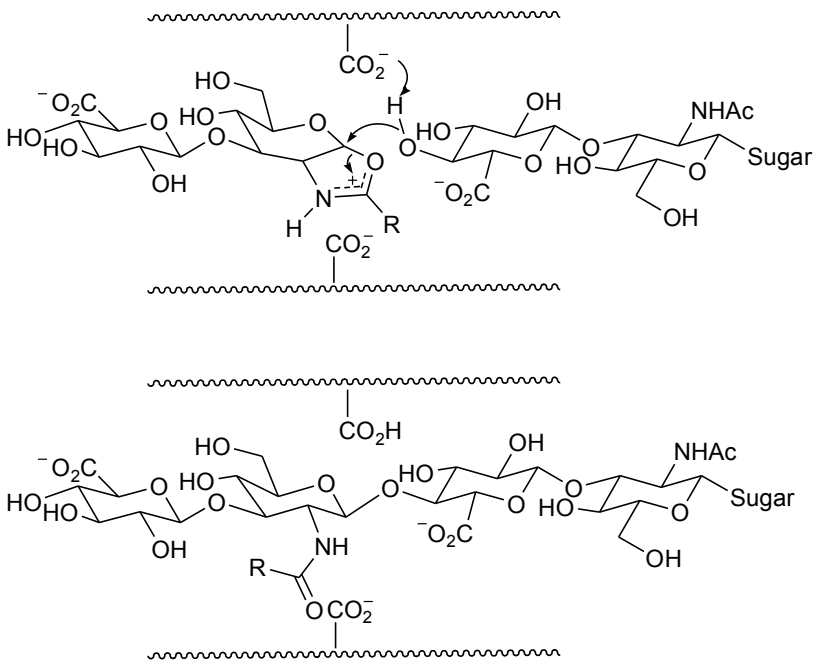
Scheme 17. DMT-MM promoted coupling of 4-amino TEMPO and a 4-amino TEMPO analogue to a GAG-disaccharide.

Overall these carboxylic acid coupling strategies have been shown to be somewhat dependent upon the nature of the amine reactive component employed and, in common with all other derivatisation strategies for GAG oligosaccharides, require tight control of reaction conditions (pH, temperature etc.) to maximise the conversion to the desired product and minimise undesired side-reactions including *N*- and *O*- desulfation. Although these methods potentially allow reaction with all the uronic acids in a GAG oligosaccharide, it is likely that the overall conformational structure will confer some selectivity on this functionalisation.[121] Finally, the inherent stability of the amide/acylhydrazide reaction products is highly desirable if they are to be used in subsequent studies.

3.2.3 Chemoenzymatic approaches

A number of recent results suggest that recombinant GAG biosynthetic,[15,129] and degradative[29] enzymes offer exciting possibilities for the selective introduction of functional labels at the non-reducing end of GAG oligosaccharides. In one example, two heparosan synthases, PmHS1 and PmHS2, from *Pasteurella multocida* have been expressed and purified using maltose-binding protein fusion constructs.[130] PmHS2 has been shown to have the ability *in vitro* to catalyse the incorporation of several unnatural UDP-donor sugar analogues, including substrates with actinide-containing uronic acids or longer acyl chain hexosamine derivatives.[130] This offers the exciting prospect that in future GAG oligosaccharide functionalisation might be controlled through the systematic introduction of modified UDP sugar donors.

In another approach, it has been shown that the ovine testicular hyaluronidase (OTH), a hydrolase, can catalyse the copolymerisation of differentially functionalised transition state analogue monomers (hyalobiuronate oxazolines) in a regio and stereoselective manner, giving rise to HA oligosaccharides bearing non-natural *N*-acyl groups in their glucosamine residues (Scheme 18).[131] By varying the comonomer feed ratio it was found to be possible to control the overall composition of the *N*-acyl groups. An enhanced control of this process might allow site-selective introduction of functionalised monomers to the GAG oligosaccharide.



Scheme 18. Proposed mechanism for OTH-catalysed polymerisation of hyalobiuronate oxazolines.

4. Applications

Reductive amination has found widespread use for the introduction of fluorescent and radioactive labels to the reducing end of GAG oligosaccharides over the past 30 years (Section 3.1.3);[72-74,102-104] these methods have been used to study a wide range of medical conditions related to GAG biosynthesis and processing. But in order to gain a deeper understanding of both normal and diseased states a detailed assessment of GAG structure and GAG-protein interactions at a molecular level is required. Some recent developments in the assessment of GAG-protein interactions using traditional and glycomics[13] based approaches (Section 4.1), and solution phase structural studies of GAGs and GAG-protein interactions (Section 4.2), are highlighted.

4.1 Assessment of protein GAG oligosaccharide interactions

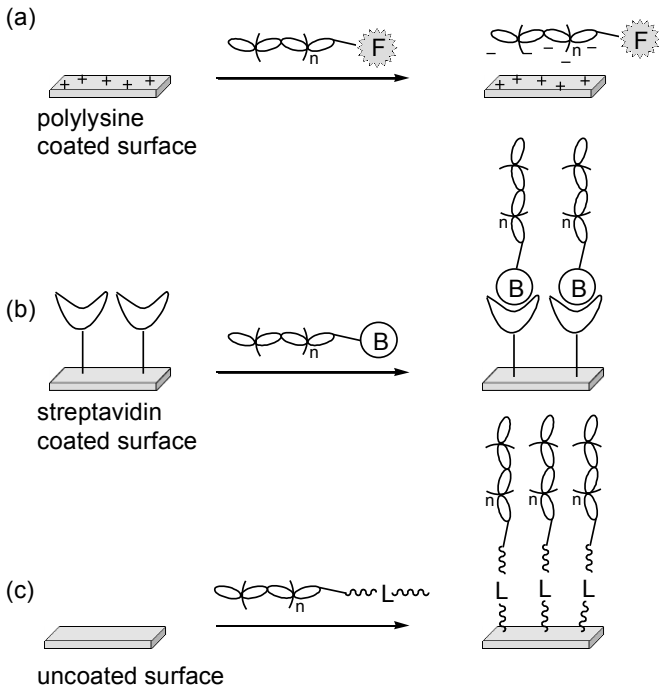
Negatively charged GAGs are very well suited for electrophoretic experiments. Their migration in a polyacrylamide or agarose gel, detected by a gel mobility shift assay (GMSA), is retarded upon binding to a protein.[74] Typically a fluorophore is attached to the GAG oligosaccharide via reductive amination to aid visualisation of the gel. GMSA may be used to deduce a minimum binding size of GAG oligosaccharide; to study the effects of GAG-sulfation patterns on binding; and to reveal differences in relative affinity of GAG oligosaccharides to different allotypes of the

same protein. In a recent study the assessment of protein binding of heparin oligosaccharides (dp2-12) using GMSA has demonstrated that a glycosaminoglycan and protein recognition site in factor H module 7 (fH~7) is perturbed by an age-related macular degeneration-linked single nucleotide polymorphism (Figure 7).[132]

Figure 7. GMSA data for His402 and Tyr402 allotypes of fH~7 with AMAC-labelled defined length heparin oligosaccharides (dp2-12). A 4-fold molar excess of fH~7 was added to 0.5 μg of AMAC-labelled oligosaccharide. The His402 is a weaker binder requiring a tetrasaccharide to register binding.

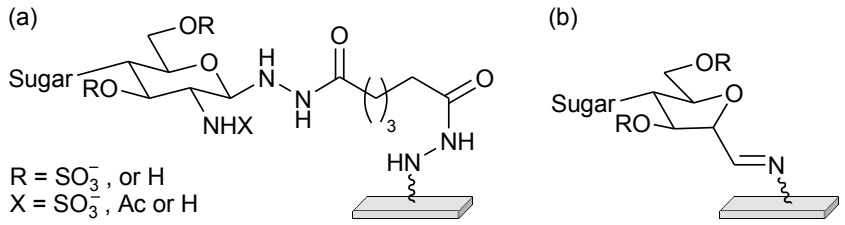
A number of techniques have been used to immobilise GAG oligosaccharides to surfaces so that these surface-bound GAGs may be used to provide a more quantitative assessment of protein-binding.[133,134] Approaches which control the orientation of the GAG oligosaccharide through attachment at the reducing end (rather than through a non-specific charge-charge immobilisation such as found with GAG oligosaccharides immobilised on a polylysine coated slide[135]) are attractive because they effectively mimic cell surface presentation of the GAG-motif, and they maximise GAG flexibility and accessibility. Modern surface technologies also allow for the use of very small quantities of the GAG oligosaccharide under investigation, as well as the determination of very weak binding affinities.

One approach to surface immobilisation is through biotinylation of the reducing end of GAG oligosaccharides which may be readily achieved using hydrazide derivatives of biotin (often with the inclusion of long-chain linkers between the biotin and hydrazide functionalities that further elevate the GAG oligosaccharide above the surface), to form the corresponding biotinyl acylhydrazone (c.f. Section 3.1.2). These biotinylated GAGs have been immobilised on streptavidin-coated surfaces and used in optical biosensor assays to determine binding characteristics of heparin-derived GAG oligosaccharides with hepatocyte growth factor / scatter factor (HGF/SF);[136] with fibroblast growth factor 2 (FGF2);[137] with glycosylated and deglycosylated FGFR;[138] and with cyclophilin B (CypB).[139] Lipid tags have also been used as an alternative to biotin for the immobilisation of GAG oligosaccharides (Scheme 8).[140]



Scheme 19. Non-covalent attachment methods used for immobilisation of GAG oligosaccharides: (a) charge-charge interactions; (b) streptavidin-biotin interaction ($K_d = 10^{-15}$); (c) neoglycolipid formation and assembly.
F = fluorophore, B = biotin, L = lipid.

One problem with a non-covalent surface attachment is that it generally requires purification of the GAG oligosaccharide (e.g. from unreacted biotin) prior to surface immobilisation; thus techniques which offer direct immobilisation of an unfunctionalised sugar to the surface can be an attractive alternative, since excess GAG oligosaccharides can simply be washed off the surface prior to the bioassay. Microtiter plates, such as those used in ELISA-type assays, coated with methyl vinyl ether–maleic anhydride copolymer (MMAC) and modified with adipic dihydrazide have been shown to give a surface which reacts directly with the reducing terminus of GAG oligosaccharides under reductive amination conditions (Scheme 9).[141] Reduction of the acylhydrazone is not a prerequisite for stability however, as it has also been shown that nitrous acid or lyase depolymerised GAG oligosaccharides immobilised through an equivalent adipic dihydrazide linker to self assembled monolayers on gold surfaces were stable to storage for several weeks when stored at 4 °C in a sealed container.[96]



Scheme 20. Covalent attachment methods for the immobilisation of GAG-oliogsaccharides: (a) acylhydrazone formation (lyase depolymerised GAG); (b) Schiff's base formation (nitrous acid depolymerised GAG).

Another direct attachment method which has found use in the immobilisation of GAG oligosaccharides is Schiff's base formation between the reducing terminus aldohexose or anhydromannose and amine-coated slides (Scheme 9). Nitrous acid depolymerised heparin-derived oligosaccharides (dp8-10) were fractionated into zero, low and high affiinity fractions on an antithrombin affinity column and then were printed on γ-aminosilane-coated glass slides.[90] GAG oligosaccharides displayed in this way maintained their relative antithrombin binding affinity. Printing of the GAG oligosaccharide to the γ-aminosilane coated slide as a solution in formamide, and then microwave heating of the slide has been proposed as an effective means of accelerating Schiff's base formation.[142]

4.2 Solution phase structural studies

X-ray crystal structures of GAG-protein complexes often display multiple binding sites, not all of which may be valid. For example, crystal structures of heparin-NK1 complexes show two modes of binding, the second of which is observed in only one of the two crystal forms and is probably the result of crystal packing.[143] One way to validate these binding sites is by the detailed study of GAG-protein interactions in solution by NMR. However, weak binding and large GAG-protein proton-proton distances are two major reasons why intermolecular NOEs fail to provide the high quality data needed for such NMR structure determination. Chemical shift perturbation analysis of ^{1}H-^{15}N HSQC spectra is therefore a method of choice for delineation of GAG binding sites in protein-GAG complexes.[132,144,145] However, this method provides qualitative information, which at best can be used to model the GAG-protein complexes. More detailed information on the solution conformation of GAG-protein complexes may be obtained through site-specific incorporation of spin labels into the oligosaccharide. The paramagnetic relaxation enhancements (PREs) of nuclei, induced by the presence of spin-labels, is well established in the NMR structure determination of biomolecules.[146] But there are only a few examples where a TEMPO-labelled

carbohydrate has been used in the application of this methodology to the study of carbohydrate-protein complexes;[147] and none of these have been GAG oligosaccharide derivatives.

A TEMPO-labelled fully sulfated heparin-derived disaccharide (Scheme 17) has been used to study the interactions of factor H, a crucial regulator of the alternative pathway of complement, with heparin.[132] The PREs of carbohydrate protons agreed well with the expected values based on an AMBER model of this molecule (Figure 8).[128] This indicates the formation of a rigid peptide bond between the 4-amino TEMPO and the COOH group of the terminal modified ΔUA. Such a well defined position of the electron radical is a distinct advantage for the interpretation of PRE data.

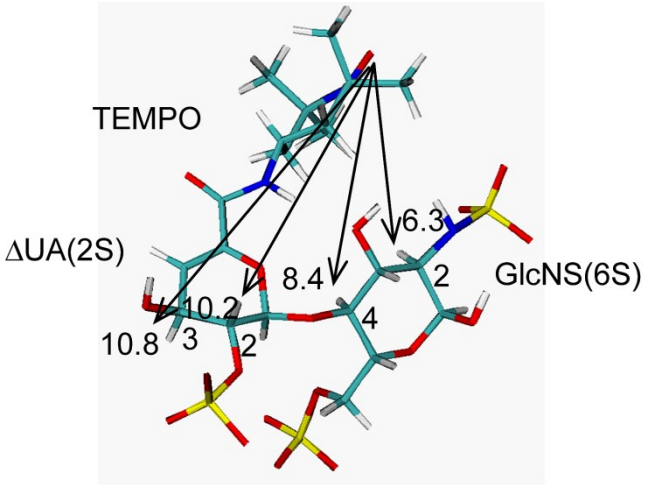


Figure 8. Solution conformation of the TEMPO-labelled disaccharide TEMPO(4NH_2)ΔUA(2S)GlcNS(6S). Selected nitroxy oxygen-proton distances are given in Angstroms.

The use of PREs in the assessment of GAG-protein complexes is exemplified by the titration of increasing amounts of this TEMPO-labelled heparin disaccharide into the protein, factor H module 7 (fH~7). Measurement of the ^{1}H relaxation times of NH protons which are significantly affected by titration (Figure 9) allows a qualitative assessment of the binding site(s) associated with this process, and indicates that at least two GAG binding sites are present in fH~7.[148] It is anticipated that a quantitative interpretation of PREs will reveal more detailed information on these binding modes.

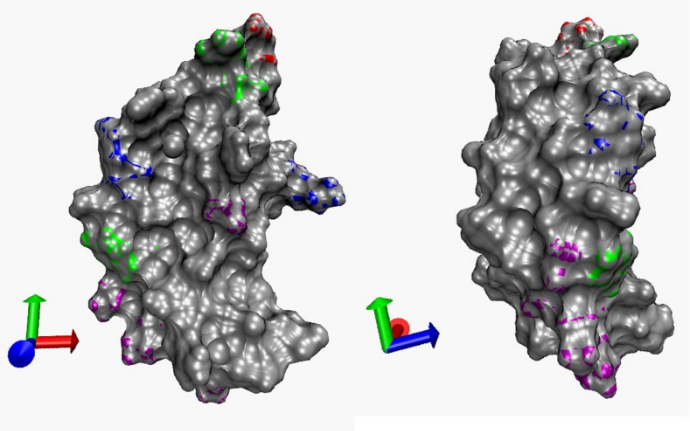


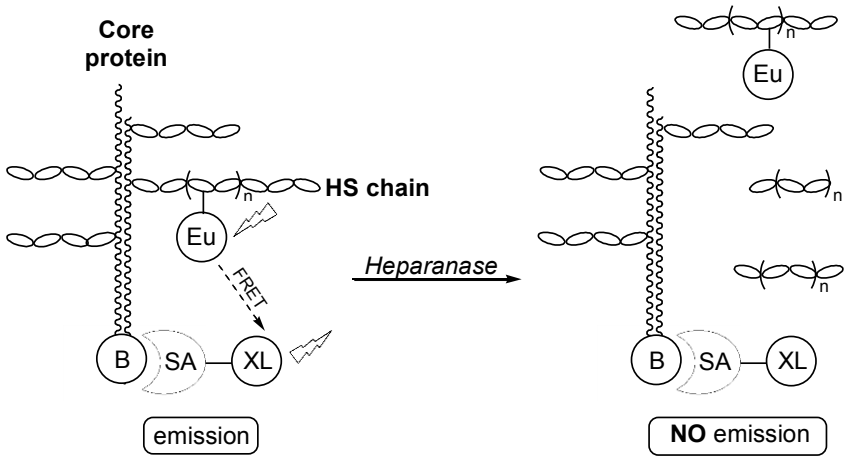
Figure 9. Surface plot of factor H~7 highlighting residues whose NH protons show largest PREs (in decreasing order from red > blue > green > magenta), indicating the existence of more than one GAG-binding site on this protein.

The use of paramagnetic species in the investigation of GAGs is not limited to the study of protein-GAG complexes; they also have great potential for the elucidation of the conformation of free oligosaccharides.

Solution phase interactions have also been extensively studied in biological systems using Fluorescence Resonance Energy Transfer (FRET). FRET generates changes in fluorescence intensity sensitive to molecular conformation, association and separation, and is widely applied to reveal spatial proximity in imaging assays.[149] Time-resolved FRET (TR-FRET) measured on the nanosecond timescale, can detect short-lived conformational states and quantitatively determine intermolecular distances on the nanometre scale.[107] TR-FRET has been used extensively to determine distances between amino acids and nucleic acids. Despite the obvious potential of the technique, FRET and TR-FRET have not been applied to the study of the solution phase conformation of GAG oligosaccharides, or the investigation of solution phase GAG-protein interactions.[150] This is largely because the installation of two fluorophores into a GAG oligosaccharide is more difficult than with proteins or nucleic acids, due to a paucity of methodology for functionalisation of the non-reducing end.

In an isolated example of TR-FRET applied to GAG-proteoglycans the acceptor fluorophore is conjugated to the protein core through biotinylation and interaction of the biotin with streptavidin labelled with a cross-linked phycobilliprotein pigment (XL665).[151] The donor fluorophore is attached to the HS chain through reductive amination of a europium cryptate diamine derivative onto a bisaldehyde generated by periodate cleavage of the oligosaccharide chain (Scheme 21). This rather non-selectively generated probe may be used in TR-FRET analysis of heparanase

activity; although as yet the assay does not give a linear correlation between the reduction in signal and degradation of the substrate. This non-linearity is most likely to be due to the structural modifications introduced to the HS oligosaccharide chain as a result of the oxidative cleavage used to generate sites suitable for the introduction of the europium FRET donor, and highlights the need for further developments in the selective conjugation of the non-reducing end of GAGs.



Scheme 21. A TR-FRET assay for heparanase activity; B = biotin, SA = streptavidin, XL = XL665, Eu = europium cryptate diamine.

5. Conclusions

If appropriate chemical/enzymatic sample preparation techniques are combined with the use of modern coupling reagents and methodologies, a wide array of selectively functionalised GAG oligosaccharides could result. These new probes will allow many aspects of GAG-protein interactions to be explored, using a range of biophysical techniques, in a manner that has not been possible to date. New areas are anticipated to include the use of fluorophores to allow TR-FRET and paramagnetic tags for NMR-based studies of GAG oligosaccharide solution conformation; NMR spin labels to determine the footprint of GAG oligosaccharides on bound proteins; and the exploitation of functionalised GAG oligosaccharide microarrays in glycomics applications. Whilst some progress has been made in this area as outlined in this review, it is clear that there is still considerable potential for further developments in this field.

Notes and references

‡ This may be achieved in an orthogonal fashion for *N*-sulfated and free amino sugars: at pH 1.5 nitrosation of the weakly basic nitrogen of *N*-sulfated sugars is achieved, most probably by H_2ONO^+; at pH 4.0 free amino sugars (which are unreactive at low pH due to protonation) react with the predominant species O_2NNO, whilst *N*-sulfated sugars are unreactive.[22]

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