**Recent Innovations in the Structural Analysis of Heparin**

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**Abstract**

Heparin, the widely used anticoagulant drug, is unusual among major pharmaceutical agents being neither single chemical entity nor a defined mixture of compounds. Its composition, while conforming to approximate average disaccharide composition or sulfation levels, exhibits heterogeneity and variability depending on the source, as well as its geographical origin. Furthermore, individual polysaccharide chains, whose physico-chemical properties are extremely similar, cannot be separated with current state-of-the-art techniques, presenting a challenge to those interested in the quality control of heparin, in ensuring its provenance and safety, and those with an interest in investigating the relationships between its structure and biological activity. The review consists of two main sections: The first is the *Introduction*, comprising (i) The History, Occurrence and Use of Heparin and (ii) Approaches to Structure-Activity Relationships. The second section is *Improved Techniques for Structural Analysis*, comprising; (i) Separation and Identification, (ii) Spectroscopic Methods, (iii) Enzymatic Approaches and (iv) Other Physico-Chemical Approaches. The ~ 60 references cover recent technological advances in the study of heparin structural analysis, largely since 2010.

**Keywords**: *Heparin, low molecular weight heparin, structure-activity, structural analysis, heparin regulation, heparin provenance*

**1. Introduction**

***1.1 History, Occurrence and Use***. The discovery of heparin is generally attributed to McLean and Howell, although there was considerable debate, not least among themselves, concerning priority. Heparin was first commercialised in the 1920's despite the observation of side-effects arising, as we now know, from impurities. By 1933, Banting and Best had developed heparin from bovine liver and this was trialled in Canada between 1933 and 1936, culminating in its successful use in humans in 1937. During this period, Jorpes in Sweden had been developing the purification process and, by 1936, an improved heparin product was available. In the 1940's, Moloney and Taylor developed a method for mass-producing heparin, which superseded the earlier one. Subsequently, heparin was used extensively as an anticoagulant in surgery and during dialysis. Later refinement of heparin has included the introduction of low molecular weight heparin (LMWH) variants, achieved using a variety of depolymerisation techniques. LMWH was produced following the discovery that the action of heparin *via* antithrombin (AT) required shorter fragments than its action through thrombin (factor IIa) and, by exploiting this selectivity, favourable clinical properties could be obtained. It was later found that a short (pentasaccharide) sequence within the heparin chains provided high affinity binding to AT and high anticoagulant activity. This line of enquiry culminated in the production of a synthetic pentasaccharide, fondaparinux, which has been commercialised. We do not intend to discuss the clinical and biochemical properties of these agents here; this has been addressed elsewhere by others far better qualified to do so ([1-3](#_ENREF_1)). Instead, we will restrict ourselves to recent developments (largely since 2010) in the structural analysis of heparin, which is itself of importance for several reasons. First, the production quality and, indeed, the identification of particular processes employed during production (especially of LMWH) can be monitored. Second, the natural variation, as well as any potential unwanted or unintended structures, can be monitored and the overall quality followed. Additionally, the complex question of structure-activity relationships, not only between heparin and proteins of the blood clotting cascade, but also many other proteins ([4](#_ENREF_4), [5](#_ENREF_5)) can be advanced. In recent years, following concerns during the 1990s about the possibility of contaminating heparin with the agents responsible for bovine encephalopathies that led to the widespread abandonment of bovine heparin as a pharmaceutical agent, commercial heparin has been obtained almost exclusively from pig intestinal mucosa, apart from some bovine material, principally in South America. However, since the contamination of the heparin supply chain with a chemically modified glycosaminoglycan (GAG) in 2008 ([6](#_ENREF_6)), there is renewed interest in diversifying the sources of heparin to the extent that bovine sources are now being considered for re-introduction into the United States market ([7](#_ENREF_7), [8](#_ENREF_8)).

Heparin is a complex mixture of polysaccharides of the GAG class, whose other members include the closely related heparan sulfate (HS), as well as chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronic acid (HA), and is one of the most important and widely-used pharmaceutical agents in current use. It is employed primarily in situations where the aim is to prevent thrombi forming, such as during surgery and blood dialysis. In its role as an anticoagulant, its mode of action is *via* several components of the blood-clotting cascade, principally antithrombin and thrombin (factor IIa) but, it also exerts action on several other components, including factors IX and XI, as well as many other proteins. At least some of these interactions with proteins seem to arise by virtue of the underlying structural similarity between heparin and the naturally occurring cell-surface polysaccharide components of proteoglycans, HS ([9](#_ENREF_9)). The principles underlying the interactions between heparin and some of these proteins, most notably AT and thrombin, have been studied extensively, employing strategies that first depolymerise the heparin into oligosaccharides, isolate the binding saccharide components and then characterise them. Using such approaches, the caveats to which have been discussed elsewhere ([10](#_ENREF_10)), the contrasting nature of interactions between heparin and AT, or with thrombin, were revealed. This early success, which led to the production first of LMWH and then to the targeted synthesis of the pentasaccharide with high activity against AT, encouraged a similar approach to be employed for the attempted isolation of analogous high-affinity oligosaccharides active with other proteins. This strategy has almost completely failed, leading some to propose an explanation of the anomalous case of AT in terms of the experimental methods used and to suggest a general property of redundancy regarding the interactions of saccharides with proteins ([11](#_ENREF_11), [12](#_ENREF_12)).

***1.2 The approach to structure-activity relationships***. To attempt to correlate the structure of heparin (and its oligosaccharide constituents) with protein binding and activity remains the ultimate aim of structure-function studies in this area. However, to do so it is now clear that the approach adopted above needs to be modified. There are a number of over-arching strategies available: One is to fractionate or, ideally, separate to homogeneity (using one or several chromatographic techniques) the components in a mixture and to determine their structure, then to correlate these with binding and activity data, noting the important point that binding does not necessarily equate with activity directly. From these data, it is then hoped that the factors determining activity will become apparent. A complementary approach attempts to conduct structural analyses on a mixture or a partially separated mixture, about which some detailed structural information is, nevertheless, available with the aim of providing some insight into the structural features of heparin responsible for activity.

Heparin is unusual among mainstream pharmaceutical agents in that it does not consist of a single chemical structure. In fact, the situation is even more complex and challenging than this simple statement would suggest, because heparin cannot be defined even as a mixture of defined substances. The reasons for this are several. In the first place, heparin is a polydisperse polysaccharide whose chain lengths and their distribution vary, not only in the source material as a function of individual animal and geographic location but, also as a consequence of subsequent processing. Next, the sequences present within heparin samples, while conforming to broad compositions in terms of the constituent disaccharides and gross sulfation position that allow, for example porcine mucosal heparin to be distinguished from bovine mucosal heparin ([13](#_ENREF_13)), also vary to some extent, leading to the property of micro-heterogeneity. Third, the processing and treatment of the heparin as it is rendered into its pharmaceutical state can also induce some additional structural changes and these depend on the process involved but, can also vary between batches, even when those processes are deemed to be identical ([14](#_ENREF_14)).

The overall challenge is therefore two-fold: *How to define 'heparin' in the first place?* Then; *how to relate the structures that comprise a heparin sample to its activity?* This second part masks further major technical obstacles which include; *the identification of the sequences present within a sample of heparin* and its usual pre-requisite; *how to separate its constituent chains* to allow that sequencing to take place. It must be stated at the outset that, with the present state of technical know-how, both of these procedures involve studying oligosaccharide fragments, rather than the full-length heparin chains and the equivalence of these approaches, especially concerning biological activity, has been questioned ([10](#_ENREF_10)). Much of this review will be devoted to surveying recent technical improvements and innovations that address these dual challenges or provide methods with which this might be undertaken.

It might also be informative to recall that the relationship between heparin oligosaccharide fragments and a very wide range of protein-binding events and activities have been found to exhibit a degree of redundancy, by which it is meant that several heparin oligosaccharide sequences appear able to elicit similar binding or activities. This is not to say, however, that all or even most sequences exhibit the same activity. A summary of the large body of data amassed to date would be that the determining properties seem to relate to a combination of charge distribution and conformation but, that these properties do not usually relate in any simple way (for example, through a straightforward consensus of particular sulfation positions or patterns) nor, in many cases, to overall charge density. It can be concluded that the physical characteristics of a binding or active oligosaccharide, while undoubtedly determined ultimately by their sequence and sulfation pattern, are not readily discernible experimentally with the present state of the art. Indeed, the apparent similarity in behaviour of many heparin oligosaccharides at the level of molecular interactions with proteins must serve as a warning of the difficulties that are likely to be encountered when attempting their separation on the basis of global properties such as charge, volume or cross-sectional area. Nevertheless, some notable progress has been made employing multi-dimensional approaches, many of which will be described below.

Undoubtedly a major step in the analysis of heparin (and other GAGs) that has blossomed in recent years is the application of mass spectrometry to structure determination. As stated above, this is usually dependent on the prior separation of oligosaccharides, although the application of ion-trap techniques can contribute to alleviating this requirement. Nevertheless, even these advances are not without their difficulties; the migration of sulfate groups during the ionisation process and the isomeric nature of several of the constituent disaccharides (hence of many oligosaccharides) being two significant examples. In the following, attempts to improve separation and conduct structural analysis, which are frequently constituent parts of the same research article, will be treated together.

**2. Improved techniques for structural analysis**

***2.1 Separation and Identification***. Separation and quantification of heparin oligosaccharides has been achieved employing reverse phase, ion-paired high-performance liquid chromatography (HPLC) combined with electrospray quadrupole-time of flight mass spectrometry (Q-TOF), which included an amine additive and 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) to improve separation ([15](#_ENREF_15)). Heparan sulfate, heparin and LMWH-derived disaccharides derivatised with 2-aminoacridone (2AA) were separated using reverse-phase HPLC with fluorescence detection and electro-spray ionization mass spectrometry (ESI-MS) ([16](#_ENREF_16)). Disaccharides from heparin, the main tetrasaccharide bearing a 3-O-sulfate group and unusual oligosaccharides arising from LMWH were separated using reverse-phase, ion paired HPLC ([17](#_ENREF_17)).

A substantial contribution to the analysis of GAGs, applied to heparin or with the potential to be applied to heparin, has been made by the group of Linhardt. These have included several improved techniques based on combinations of chromatography and mass spectrometry, including ultra-high performance (UHP) HPLC Q-TOF analysis of LMWH including a profiling of LMWHs in which up to 120 oligosaccharides were identified and assigned ([18](#_ENREF_18)), liquid chromatography (LC) tandem MS ([19](#_ENREF_19), [20](#_ENREF_20)), hydrophobic interaction liquid chromatography (HILIC) FT MS ([21](#_ENREF_21), [22](#_ENREF_22)), LC-MS analysis of oligosaccharides resistant to heparinase II (hII) enzyme digestion ([23](#_ENREF_23)), GAG analysis employing capillary zone electrophoresis-laser induced fluorescence (CZE-LIF) with 2AA labelling ([24](#_ENREF_24)) and 2AA labelling for fluorescent detected LC-MS ([25](#_ENREF_25)). Finally, a promising technique, with the potential to differentiate oligosaccharides with the same mass to charge ratios, a long-standing and fundamental problem in the field, has been developed ([26](#_ENREF_26)).

In other work, the effect of the ion-pairing reagent and eluent pH on the separation of disaccharide components by reverse phase chromatography was investigated and optimum resolution was obtained under low pH conditions ([27](#_ENREF_27)). Using ultra-performance gel permeation chromatography (GPC) combined with Q-TOF MS, LMW heparin was fractionated into 70 components and further studied using CZE ([28](#_ENREF_28)). A mass spectrometry study of disaccharides from chemically modified heparins, detected 1,6-anhydro residues and demonstrated an unusual periodate cleavage of amino sugar residues ([29](#_ENREF_29)).

Capillary electrophoresis (CE) has been used to conduct a total GAG analysis ([24](#_ENREF_24)). The numerous methods for separating heparin-derived oligosaccharides by CE have been reviewed elsewhere ([30](#_ENREF_30)). Of particular interest is CE under conditions amenable to subsequent analysis by MS ([31](#_ENREF_31)). One further approach, employing CE that has been applied to CS but, which also holds promise for improved heparin disaccharide analysis employs fluorescence resonance energy transfer (FRET) based on the conjugation of a quantum dot (linked *via* streptavidin) as donor and cyanine-5 (cy5) as acceptor ([32](#_ENREF_32)).

***2.2 Spectroscopic methods***. One area in which significant advances have been made in recent years concerns the study of the 15N nucleus by nuclear magnetic resonance (NMR) spectroscopy in GAGs, including heparin and model compounds. This aspect of NMR spectroscopy provides information that is complementary to other NMR techniques (i.e. detecting 1H and 13C) and other structural techniques in general. The principal investigators in this field have been the group of Larive, who have detected 1H and 15N resonances of the sulfamate (-NHSO3-) group which is situated at position-2 of glucosamine residues, in monosaccharides and the pentasaccharide ([33](#_ENREF_33), [34](#_ENREF_34)), then heparin and HS ([35](#_ENREF_35)), going on to study solvent exchange with the N-H proton to detect a persistent hydrogen bond between the sulfamate N-H proton and the adjacent 3-O-sulfate group in the pentasaccharide ([36](#_ENREF_36)), which has been modelled ([37](#_ENREF_37)). They have also pioneered the use of long-range 1H-15N correlations on monosaccharides to show that the 6-O-sulfate does not significantly affect the environment of the nitrogen ([38](#_ENREF_38)) and have improved the sensitivity of the technique ([39](#_ENREF_39)). The same group has also employed NMR to follow the enzymatic degradation of heparin ([40](#_ENREF_40)) and have constructed a high-sensitivity NMR micro-coil, which allowed minor components to be detected, including two previously unreported tetrasaccharides from heparin ([41](#_ENREF_41)). Advances have also been made using ion mobility mass spectrometry ([42](#_ENREF_42)), electron ionisation (EI) ion trap mass spectrometry ([43](#_ENREF_43)) as well as in improved separation of oligosaccharides employing graphitized carbon columns followed by ESI-MS ([44](#_ENREF_44)).

 Other notable findings have included the identification of a heparin dodecasaccharide containing two antithrombin binding sites ([45](#_ENREF_45)), an antithrombin-binding octasaccharide with an additional 3-O-sulfated glucosamine residue in the active pentasaccharide ([46](#_ENREF_46)) and the study of the conformation and activity of 1,6-anhydro sugars identified in antithrombin-binding octasaccharides ([47](#_ENREF_47)).

In a distinct set of approaches, NMR statistical correlation spectroscopy has been employed to extract structural details directly from the heparin mixture, without purification. The tri-sulfated glucosamine residue has been identified within both the conventional pentasaccharide, but also at the non-reducing terminal of heparin chains. In addition, IdoA2S residues linked to glucosamine 6-sulfate (free-amine) adjacent to the linkage region ([48](#_ENREF_48)) were observed. The production of some heparin preparations, such as low molecular weight Enoxaparin, the generic form of Lovenox, involves the application of base treatment of the benzoyl ester of heparin and this basic step can introduce several additional modifications into the structure. These include a small amount of epoxide, formed between positions -2 and -3 in formerly 2-O-sulfated iduronate 2-O-sulfate residues ([49](#_ENREF_49)) and formation of 1,6-anhydro-amino sugars (both GlcN and ManN, the latter of which arises from base-catalysed epimerisation ([50](#_ENREF_50)). All of these modifications, as well as varied levels of the linking tetrasaccharide containing fragment, GlcA-Gal-Gal-Xyl-ser, can be detected in samples of commercial LMWH employing a combination of NMR (in this case, using heteronuclear single quantum coherence (HSQC) spectra) and multivariate analysis techniques of a library of such products ([14](#_ENREF_14)). This analysis overlaps to some extent with the identification of contaminating species within the heparin samples. Indeed, in many respects, the challenge of separation and/or distinguising characteristic signals is similar.

The most complete description of oligosaccharides obtained from heparin has been achieved by Hricovini, who has combined NMR with molecular simulations, studying the effects of solvent and counter ions ([51](#_ENREF_51)), a trisaccharide repeat of heparin ([52](#_ENREF_52)) and most recently, a study of the pentasaccharide employing density functional theory (DFT) and NMR, identifying new hydrogen bonds ([53](#_ENREF_53)).

Since heparin carries a substantial negative charge, it can be made to migrate in an electric field, which is the basis of electrophoresis techniques (gel electrophoresis and forms of CE), which have been mentioned above. Under normal conditions there are no, or only very few, free amino groups present in the molecules, lending the macromolecule an extremely acidic nature and very low pI. However, there are certain circumstances in which an increased positive charge can be introduced. This can occur either by the binding of some cationic species (as was achieved in early work by Nader and by Johnson ([54](#_ENREF_54), [55](#_ENREF_55)) or, through the extensive removal of N-sulfates and/or N-acetyl groups (both of which are selective and reversible by chemical means) to expose free amino groups which, under low pH conditions, are protonated, hence, positively charged, resulting in zwitterionic species with low net pI. This is the basis upon which Holman *et al*., achieved focusing of heparin oligosaccharides using low pH gradient isoelectric focusing (IEF) following earlier fractionation by GPC and high performance anion exchange chromatography (HPAEC) ([56](#_ENREF_56)).

***2.3 Enzymatic approaches***. In a separate line of research, the Liu and Linhardt research groups have pioneered the development of enzymatic approaches to the enzymatic synthesis of heparin oligosaccharides while, strictly speaking, not an analytical technique *per se*, provides tools (defined oligosaccharides) which promise to be of considerable significance experimentally as well as serving as analytical standards ([57-59](#_ENREF_57)) ([60-62](#_ENREF_60)).

***2.4 Other Physico-Chemical Approaches***. The group of Dubin has made important contributions to the discussion concerning the nature of interactions between heparin and proteins, arguing against the kind of exquisite specificity proposed earlier for the interactions between proteins and heparin ([63](#_ENREF_63)) and, for example, showing that charge-charge interactions account for as little as 30% of the free energy of binding ([64](#_ENREF_64)). These findings resonate with a recent theoretical report stating that positively charged amino acids (Arg and Lys) are poor predictors of heparin binding sites on protein surfaces, while the uncharged side chains of Gln and Asn were posited as important for their ability to hydrogen bond ([65](#_ENREF_65)). The geographical origin of heparin, which is of particular concern in light of problems with provenance, has been examined through analysis of stable isotopes, revealing that the content of 13C and 18O isotopes provided good overall ability to differentiate its origin ([66](#_ENREF_66)). The possibility of de-N-sulfation in heparin samples, arising from heating and/or exposure to acidic conditions or solvents has also been assessed, employing labelling of the resultant free amine groups and disaccharide analysis employing HILIC MS ([21](#_ENREF_21)).

We have included these latter miscellaneous findings because, they suggest that our view of those features of the heparin chain that are currently considered important for protein binding and activity need to be continually questioned, and this will influence the nature of the information we seek concerning heparin structure in the future.

**3. Conflict of interest statement**

The authors declare no competing financial interest.

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