Hydrolytic Degradation of Heparin in Acidic Environments; NMR Reveals Details of Selective De-Sulfation.

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ABSTRACT

Heparin, is a complex glycosaminoglycan, derived mainly from pig mucosa, used therapeutically for its anticoagulant activity. Yet, owing largely to the chain complexity, the progressive effects of environmental conditions on heparin structure have not been fully described. A systematic study of the influence of acidic hydrolysis on heparin chain length and substitution has therefore been conducted. Changes in the sulfation pattern, monitored *via* 2D-NMR, revealed initial de-N-sulfation of the molecule (pH 1/ 40 °C) and unexpectedly identified the secondary sulfate of iduronate as more labile than the 6-O-sulfate of glucosamine residues under these conditions (pH 1/ 60 °C). Additionally, the loss of sulfate groups, rather than depolymerisation, accounted for most of the reduction in molecular weight. This provides an alternative route to producing partially 2-O-de-sulfated heparin derivatives that avoids using conventional basic conditions and may be of value in the optimisation of processes associated with the production of heparin pharmaceuticals.

KEYWORDS:

1. Heparin
2. Acid stability
3. ¹H- and 2D NMR
4. De-sulfation
5. Hydrolytic degradation

INTRODUCTION

Heparin is a complex linear polyanionic polysaccharide found in the mast cells of higher animals and belongs to the heterogeneous family of glycosaminoglycans (GAGs).¹⁻³ Heparin closely resembles the related GAG, heparan sulfate, which is commonly expressed on the surface of cells in all higher animals and is involved in the regulation of many developmental and cell-signalling processes, whereas heparin often serves as a convenient experimental proxy through interactions with a multitude of proteins.⁴⁻⁶

Owing to its non-template driven biosynthetic pathway,⁷⁻⁹ heparin exhibits variation in composition and polysaccharide chain length, both between distinct animal or tissue sources (e.g. porcine intestinal mucosa *vs* bovine lung), but also within samples from individuals of the same species. The highly sulfated and uronic acid-rich polysaccharide chains of heparin are based predominantly on 1,4 linked, alternating α -D-glucosamine (GlcN) $\rightarrow$ α -L-iduronate (IdoA) units, and less commonly, on α -D-glucosamine (GlcN) $\rightarrow$ β -D-glucuronate (GlcA) residues. Each of these disaccharide residues have several potential sites of sulfation. These include position 2- (2S-) of iduronate and positions 6-, 3- and 2- of glucosamine (6S-, 3S- and NS-, respectively). Amongst these, the N-sulfate is the most prevalent, whereas the O-sulfate at position-3, although found more rarely, plays a significant role in the anticoagulant activity of the molecule. The possible variations in the elementary units and different potential combinations of sulfation patterns contribute to the substantial heterogeneity of heparin. Amongst all the possible structural modifications (at each disaccharide building block), IdoA(2S)(1 $\rightarrow$ 4) GlcNS(6S), shown in **Figure 1**, constitutes from 60 to 90% of the heparin structure, while other disaccharides make up the remainder.^{6,11,12}

Figure 1. The major heparin disaccharide repeating unit of $\rightarrow 4$)- α -L-iduronic acid-(1 $\rightarrow$ 4)- α -D-glucosamine (1 $\rightarrow$ (left) and the minor disaccharide repeat unit of $\rightarrow 4$)- β -D-glucuronic acid-(1 $\rightarrow$ 4)- α -D-glucosamine (1 $\rightarrow$ (right).

It has been established that a major contribution to the biological activity of heparin follows from electrostatic interactions between the negatively charged sulfate groups densely populated throughout the polymer chain, brought into appropriate geometric proximity with basic amino acid residues (arginine, lysine and at appropriate pH values, also histidine) of heparin-binding proteins.^{5,7,10} The selectivity of the binding is further adapted to control numerous important biological processes, among which the blood coagulation has been examined with the greatest attention^{3,13,14}. The ability of heparin to modulate blood clotting, arises principally from its interactions with two proteins; antithrombin (ATIII) and thrombin (factor IIa) but, importantly, the nature of these interactions at the molecular level differs considerably. While interactions with thrombin require an extended stretch of heparin with little requirement for particular heparin sequences,¹³ those between heparin and ATIII involve higher affinity interactions, conditional upon pentasaccharide sequences containing the low-abundance tri-sulfated glucosamine residue,¹⁵ for subsequent action on factor Xa.

Since the discovery of heparin by McLean in 1916, numerous studies have been undertaken to understand the relationship between the chemical structure and the pharmacological activity of

heparin.^{4–6,8,16–18} The key points are that the activity of heparin depends on its structure and can be manipulated towards specific pharmaceutical agents of different functionality (*e.g.* low molecular weight heparins). Therefore, it is rather surprising that the chemical stability of heparin, in particular, the effect of stressing conditions on its detailed substitution patterns, have been explored to a lesser extent. Especially considering the dependency of the commercial heparin from animal sources and the range of conditions that are applied during its purification¹⁹ and in the production of low-molecular weight heparin.^{20,21}

Among potential chemical modifications, hydrolysis is one of the major processes affecting the chemical stability of pharmacologically active substances.^{23–25} In many pharmacologically active polysaccharides, it leads to cleavage of glycosidic bond(s), changes in molecular integrity and can therefore cause deviation from drug specifications.^{23–25} In the case of heparin, the possibility of changes to the sulfation pattern must also be considered, since these groups are susceptible to modification at both acidic and alkaline pH,^{2,26,27} while being critical to maintaining the pharmacological activity of the polysaccharide. Therefore, it is crucial to identify conditions that should be avoided, in order to maintain the anticoagulant activity of heparin but, also provide a strategy to manipulate the sulfation pattern to generate “non-anticoagulant” derivatives with differing functionalities

Much fundamental knowledge concerning the chemical stability of heparin in mild acidic conditions is based on Danishefsky’s work (1960), which was an early examination of the hydrolytic behaviour of heparin in such an environment.²⁸ Danishefsky focused on hydrolytic sulfate loss, the order of de-sulfation and the relative resistance of the glycosidic linkages to hydrolytic scission. Fifteen years later, Inoue and Nagasawa (1976) extended the scope of degradative conditions to both hydrolytic and solvolytic environments²⁹ adopting the concept of

preferential sulfate loss, introduced by Danishefsky, to establish the order of de-N-/ de-O-sulfation. These reports, along with several viscosity studies from the 1970s,^{30,31} as well as the later work of Jandik *et al.*, (1996)³², constitute most of our fundamental knowledge regarding the hydrolytic stability of heparin. In fact, to the best of our knowledge, the resistance of heparin to glycosidic scission and the susceptibility of the sulfo-groups to hydrolytic conditions have not been explored in detail since then. On the other hand, solvolysis has been the key to the deliberate chemical modification of heparins^{33–35} and established that the order of removal of sulfate groups did not proceed in such a manner as to allow their entirely selective removal. While de-N-sulfation occurred under sufficiently mild conditions to allow its use as a selective reaction, the removal of 6-O and 2-O sulfates was less selective.³³ Subsequent studies were able to devise the means of achieving higher selectivity for the removal of 6-O-sulfation,^{26,34} while selective removal of 2-O-sulfate groups relied on the prior formation of a 2,3-epoxide intermediate³⁶ and its subsequent opening under harsh basic conditions, that also resulted in extensive depolymerisation and other modifications.³⁷

Herein, to mark 60 years since Danishefsky's pioneering work, the process of hydrolytic degradation of heparin has been re-examined under a range of acidic aqueous environments (pH 1 - 6) and temperatures (40, 60 & 80 °C) over time (up to 48 h). This research complements and updates the acid-catalysed degradation mechanism of heparin, introduced by Jandik and co-workers (1996),³² and provides evidence of the hydrolytic desulfation order, contrasting with that observed under solvolytic conditions.^{29,34}

This work contributes to a deeper understanding of the structural changes of heparin and highlights the impact of external conditions on intramolecular modifications. From the practical perspective, the results of our study have the potential to be applied to improve the control of

manufacturing processes and optimise the storage conditions of products containing pharmaceutically active heparin. Furthermore, they provide an alternative route to certain modifications of the heparin molecule that may contribute to the preparation of new heparin-based derivatives.

EXPERIMENTAL

2.1 Materials

All reagents used in this research were of analytical grade or higher. All solutions were prepared in Milli-Q ultrapure water of $\geq 18.2 \text{ M}\Omega\cdot\text{cm}$ resistivity at 25°C .

Trifluoroacetic acid (99%), sodium hydroxide, potassium chloride, sodium citrate, citric acid, 19:1 acrylamide/bis-acrylamide dry powder blend (A2917), Trizma base ($\geq 99\%$, crystalline), MES monohydrate ($\geq 99\%$), N,N,N',N'-tetramethylethylenediamine (TEMED, $\sim 99\%$), 7-amino-1,3-naphthalenedisulfonic acid monopotassium salt monohydrate (ANDSA, $\geq 98\%$, glycerol ($\geq 99\%$), Azure A chloride, sodium acetate ($\geq 99\%$), N-acetylglucosamine (GlcNAc), sodium carbonate ($\geq 99.0\%$), sodium bicarbonate (99.5%), certified multi-anion (F^- , Cl^- , Br^- , NO_3^- , PO_4^{3-} , SO_4^{2-}) standards ($10 \text{ ppm} \pm 0.2\%$ per each anion) acetate and citrate anion standards (1000 ppm), deuterium oxide (D_2O , $\geq 99.96\% \text{ D}$) and deuterium chloride (DCl , 35 wt.% in D_2O , $\geq 99 \text{ atom}\%$ D) were purchased from Sigma-Aldrich (Gillingham, UK).

Concentrated hydrochloric acid (12.1 M), ammonium acetate, sodium azide, bovine serum albumin (BSA, M_w 66 kDa), ethylene glycol (99.5%), ammonium persulfate (APS, $\geq 99\%$), formamide (99.5%), tris(hydroxymethyl)aminomethane acetate salt (99%,), LDS sample buffer, sodium hydroxide (50% w/v,) galactosamine (GalN), glucosamine (GlcN), fucose (Fuc), glucuronic acid (GlcA) and galacturonic acid (GalA) were purchased from Fisher Scientific (Loughborough, UK).

Glucosamine-2-N-6-O-disulfate (GlcNS(6S)) and glucosamine-2-N-3-O-disulfate (GlcNS(3S)) were purchased from Dextra Laboratories Ltd. (Reading, UK). Glucosamine-2-N-sulfate (GlcNS) was purchased from Carbosynth Limited (Compton, UK) and Iduronic acid (IdoA) was purchased from Synthose (Ontario, Canada).

2.2 Heparin sample preparation

The sodium salt of porcine mucosal heparin ($M_{w,0} \approx 20,000$ g/mol, $M_w/M_n = 1.1$) was provided by LEO Pharma (Cork, Ireland/ Ballerup, Denmark) and used without any further purification. The aqueous solution of heparin was prepared by dissolving 10 g of accurately weighed oven-dried heparin sodium salt (48 h/ 60 °C) in 1000 mL of ultrapure water with stirring for 12 h. This solution is referred to as ‘heparin stock’ (final concentration, 10 mg/mL).

2.3 Degradation of heparin in acid environments

2.3.1 Partial acid hydrolysis: the experimental procedure was a slightly modified version of the method previously described³⁸. The stock solution of heparin sodium (100 mL) was transferred into sealable glass bottles (250 mL) and buffered as follows: pH 1 (HCl – KCl), pH 2 (HCl – KCl), pH 3 (citric acid - sodium citrate), pH 4 (citric acid - sodium citrate), pH 5 (citric acid - sodium citrate) and pH 6 (citric acid - sodium citrate). The final concentration of each buffer was adjusted to 0.1 M. Buffered samples were further incubated in a water bath at 40 °C, 60 °C and 80 °C (separate runs) and pH controlled. Aliquots (5 mL per sample) were collected at various time points up to 48 hours, immediately cooled-down in an ice-water bath and neutralised with 1.0 M NaOH to pH 7- 7.5. Next, the neutralised hydrolysates were diluted with ultrapure water to a final concentration of 5 mg/mL, divided into 2.5 mL aliquots and stored at -20 °C for further analysis.

2.3.2 TFA hydrolysis: The heparin sodium salt was hydrolysed in 2.0 M aqueous solution of trifluoroacetic acid (TFA) to prepare the hydrolytic oligosaccharide standards^{39,40}. The progress of

reaction was monitored by TLC (data not shown)⁴¹. The oven-dried sample (25 mg) was dissolved in 2.5 mL of TFA to a final concentration of 10 mg/mL. Degradation was carried out in pressure tubes at 100 °C for 24 hours. Aliquots were removed at various time points and cooled in an ice bath to stop the hydrolysis. The excess was then removed at 40 °C, under a constant stream of nitrogen (TurboVap® LV Caliper Life Sciences, Waltham, Massachusetts, USA). Dried samples were re-dissolved in water, neutralised with 1.0 M NaOH and freeze-dried (Christ Alpha 2-4 LSC freeze- dryer, Martin Christ, Osterode am Harz Germany). Lyophilized samples were weighed and further re-dissolved in ultrapure water; to a final concentration of 5 mg/mL. Samples were stored at -20 °C until further analysis.

2.4 Determination of the molecular weight

The heparin hydrolysates were analysed at a concentration of 5 mg/mL. Each analytical sample was filtered through a 0.45 µm regenerated cellulose (RC) syringe filter (17 mm, Target2™, Thermo Fisher Scientific, Paisley, UK), prior to injection (100 µL). Monodisperse bovine serum albumin (BSA) standards were prepared in identical solvents to those of experimental samples, at a concentration of 2 mg/mL and used for normalization of the system.

Hydrolysates of heparin were separated on TSK G4000 SW_{XL} and TSK G3000 SW_{XL} columns (Tosoh Bioscience, Tokyo, Japan), protected by similarly packed guard column and eluted with a 0.1 M NH₄OAc + 0.05% (w/v) NaN₃ mobile phase at a flow rate of 0.6 mL/min and temperature of 30 °C. Samples were detected using MALS (DAWN 8+) and refractive index (Optilab T-REX) detectors (Wyatt Technology Corporation, Santa Barbara, USA). Data for molecular weight determinations were analyzed using Astra software v. 6.1.5 (Wyatt Technology Corporation, Santa Barbara, USA). Refractive index increments (dn/dc) of 0.131 mL/g for heparin⁴² and 0.185 mL/g for BSA⁴³ were used respectively in data processing.

2.5 PAGE as a reducing ends assay

The PAGE gel casting and imaging procedure was based on two protocols, described previously^{37,38}. First, selected heparin hydrolysates (2.5 mL) were loaded onto PD-10 column, prepacked with SephadexTM G-25 Medium (GE Healthcare Life Sciences, Little Chalfont, UK), and desalted with water. The eluates were collected, freeze-dried, accurately weighed and re-dissolved in water to a final concentration of 5 mg/mL (per hydrolysate). The desalted solutions (20 μ L) were again lyophilized, fluorescently tagged (25 μ L) with a saturated formamide solution of ANDSA (80 mg/mL) and incubated for 16 hours at 25 °C. Tagged samples were loaded onto 33% acrylamide tris-acetate gels (home-casted). Electrophoresis was performed in Tris-MES buffer at a constant voltage (240 V) for ~3 hours at 4 °C. The ANDSA tagged-heparin oligosaccharides (dp 2 – 10), were mixed with LDS sample buffer containing phenol red and Coomassie G250 dyes, then loaded into separate wells to track the separation progress. These oligosaccharide standards also served as a separation reference ladder. The electrophoresis was terminated when the red marker reached about 5 cm from the gel bottom and gels were immediately viewed on a UV transilluminator (λ_{max} = 302 nm) with a gel imaging camera (G-BOX fluorescence system, Syngene, Cambridge, UK) and recorded using GeneSnap software (Cambridge, UK). The electrophoretic runs were duplicated.

2.6 Monosaccharide analysis

Monosaccharide standard mixtures (galactosamine (GalN), glucosamine (GlcN), fucose (Fuc), glucuronic acid (GlcA), galacturonic acid (GalA), N-acetylglucosamine (GlcNAc), glucosamine-2-N-6-O-disulfate (GlcNS(6S)), glucosamine-2-N-3-O-disulfate (GlcNS(3S)), glucosamine-2-N-sulfate (GlcNS) and iduronic acid (IdoA)) were prepared at different concentrations of 0.5, 1.0, 5.0, 10.0, 25, 50, 100 ppm per saccharide. Each monosaccharide was also prepared at an individual

concentration of 50 ppm, to confirm the retention time in the mixture. Heparin hydrolysates were diluted to 500 ppm and spiked with internal standard of fucose (50 ppm). Each analytical sample was filtered through a 0.45 μm regenerated cellulose (RC) syringe filter (17 mm, Target2™, Thermo Fisher Scientific, Paisley, UK), prior to injection.

The analysis was performed using a Dionex ICS-3000 Ion Chromatography System (HPAEC, Dionex Corporation, CA, USA) with Reagent-Free Eluent Generation. The analytical compartment was composed of Eluent Organiser with Dual Pump Channel, Detector Module, Autosampler and Pulsed Amperometric Detector (PAD) with disposable gold working electrode. Separation was carried out using a CarboPac PA20 analytical column (3 x 150 mm), protected by similarly packed guard column (3 x 30 mm, BioLac™, Thermo Fisher Scientific, Paisley, UK) at room temperature. Mobile phase was delivered at 0.3 mL/min, with isocratic flow of mobile phase A (15 mM NaOH) for the first 15 min followed by linear gradient of mobile phase B (150 mM NaOAc + 15 mM NaOH) for the next 45 min²⁵. The details of method optimization are provided in Supporting Information. Helium was applied as a carrier gas at a pressure of 50 - 60 psi. The injection volume was set to 25 μL . The chromatograms were recorded with Chromeleon software v. 6.8 (Thermo Fisher Scientific, Paisley, UK). All samples were analysed in at least duplicate.

2.7 Quantitative analysis of inorganic anions

The solutions of multi-anion standards (F^- , Cl^- , Br^- , NO_3^- , PO_4^{3-} , SO_4^{2-}) and sodium acetate (separate) were prepared from certified stocks at concentrations of 0.1, 0.5, 1.0, 2.0, 3.0, 5.0 ppm of each anion. The solution of sodium citrate was prepared at a single concentration of 10.0 ppm. Heparin hydrolysates and stock were diluted to 10.0 ppm and filtered through 0.45 μm polyethersulfone membrane (PES) syringe filters (13 mm, Azure, Gilson Scientific, UK).

The analysis was performed on Dionex ICS 1500 Ion Chromatography System (HPAEC) with Self Regenerating Suppressor (SRS) and DS6 conductivity detector (CD, Dionex, Camberley, UK). The separation was carried out on Dionex™ IonPac™ AS14 IC Column, guarded by similarly packed column (Thermo Fisher Scientific, Hemel Hempstead, UK). Mobile phase composed of 3.2 mM sodium carbonate and 1.0 mM sodium bicarbonate was delivered at 1.2 mL/min isocratic flow at 30 °C. The injection volume was set to 25 µL with acquisition time of 15 min. The chromatograms were recorded with Chromeleon software v. 6.8 (Thermo Fisher Scientific, Paisley, UK). The standards and hydrolysates were analysed in triplicate. The calculations were based on replicated analysis using the standard curves. The data collected from chromatographic methods were copied to Microsoft Excel in numerical format and further analysed and re-plotted in GraphPad Prism v. 8.4.1 (GraphPad Software, San Diego, CA, USA).

2.8 Analysis of structural changes

NMR spectroscopy has emerged as the most informative technique for examining the detailed structure of intact polysaccharides. In the case of GAGs, the positions at which the ^1H and ^{13}C resonances appear on the frequency scale (i.e. their chemical shift positions) are sensitive to the local chemical environment and are influenced by a range of factors, including the neighbouring groups like hydroxyl, carboxylate and amine, glycosidic oxygen and O- or N-sulfate substitution. In particular, the chemical shift positions of ^{13}C and ^1H nuclei proximal to hydroxyl and amine groups are considerably shifted downfield, that is, to higher chemical shift values, in both ^1H and ^{13}C dimensions by substitution with the strongly electron-withdrawing sulfate group. Conversely, the removal of a sulfate group results in an upfield shift (*i.e.* lower chemical shift values).

In heparin, the NMR shift patterns have been studied systematically and considering variants of each combination of sulfation and de-sulfation in IdoA and GlcN residues. The redeployment of characteristic signals has been recognised (and assigned) in regard to sulfation pattern changes. This enables the extent and position of de-sulfation in the hydrolysed heparin fractions to be determined readily from the spectra.

To analyze structural changes NMR measurements were performed on Bruker Advance Neo 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with Bruker Prodigy Cryoprobe and a 24-position SampleCase autosampler. The data were recorded automatically and manually phased and baseline-corrected in TopSpin 4.0.6 software (Bruker Biospin, Rheinstetten, Germany).

Heparin sodium (50 mg as standard) and desalted (see **section 2.4**) heparin hydrolysates (10- 20 mg) at pH 1 (40, 60 and 80 °C; 24 h and 48 h) were repeatedly lyophilized against D₂O to remove the exchangeable protons. The thoroughly dried samples were redissolved in 0.6 mL D₂O mixed with acetone (reference standard, 10 µL/100 mL D₂O) and transferred to precision grade NMR tubes (OD 5 mm, borosilicate glass). The 1D ¹H spectra were recorded at 60 °C (333 K) with a standard pulse program, 2 sec delay and 16 scans with the total acquisition time of 2.75 sec. ¹³C analysis was approached with 2 sec delay, 2048 scans and total acquisition time of 1.6 sec. The HSQC spectra were recorded with carbon decoupling, 1.44 sec delay and 2 scans, with ¹J_{C-H} constant set to 150 Hz.

RESULTS AND DISCUSSION

3.1 Effect of acidic conditions on molecular weight of heparin.

Molecular weight has been recognized as one of the most important characteristics underpinning the functionality of polysaccharides.^{44,45} In the case of heparin, the specific medical response is

directly associated with its molecular weight and chemical structure ^{13,15,16}. Therefore, the impact of the applied experimental conditions on the molecular weight was considered as a natural starting point for stability studies.

Heterogeneous polysaccharides, like heparin, can be characterized by means of number average molecular weight (M_n) and/or weight average molecular weight (M_w). In this study, SEC-MALS-RI was applied to follow the change of M_w of thermally stressed heparin (40, 60, 80°C) in acidic environments (pH 1- 6) for up to 48 hours. The collected results, plotted as a function of time in **Figure 2**, illustrate the inverse relationship between the molecular weight loss and pH, while the rate of molecular weight degradation increased with temperature. The data show that the heparin molecule is stable between pH 4 and 6 at all studied temperatures. At pH 1, the molecular weight decreased by 17%, 18% and 24% at 40 °C, 60 °C and 80 °C, respectively, whereas under milder conditions of pH 2 and pH 3, the molecular weight has dropped by respectively 10%, 15%, 21% and 2%, 10%, 13%, at the studied temperatures (**Figure 2**). Based on previous literature, ^{28,32} the initial resistance of heparin to mild acidic environment was expected, however, the 24% molecular weight loss at elevated temperature and low pH (80 °C/ pH 1) can be considered relatively modest, considering that similar conditions have been applied for the preparation of heparin oligomers and low molecular weight heparins.^{20,21} Bearing this in mind, the extent of glycosidic bond scission was examined further.

Figure 2. Average weight molecular weight of heparin at (a) 40 °C, (b) 60 °C and (c) 80 °C plotted as function of time at pH 1- 6. The averaged uncertainty defines the 95% confidence interval. ● pH 1, ● pH 2, ● pH 3, ● pH 4, ● pH 5, ● pH 6

3.2 Effect of acid conditions on glycosidic bonds; an assay for new reducing ends.

The appearance of new reducing ends was previously used as direct evidence of polysaccharide degradation.^{32,46,47} Thus, to assess the extent of chain scission, PAGE electrophoresis with fluorescent reducing end labelling was employed. Only samples which showed considerable

molecular weight loss (based on SEC-MALS-RI data – in **section 3.1**) were chosen for PAGE analysis, that is, heparins hydrolysed at pH 1- 3, at 40 °C, 60 °C and 80 °C. The ANDSA-labelled hydrolysates (100 µg, various time-points) were loaded onto the separate acrylamide gels, submitted to electrophoresis, and visualized. Exemplar gel images are shown in **Figure 3**. It was confirmed that treatment at pH 2 and pH 3 did not result in any visible bands at any hydrolytic time-point, at any of the three temperatures (2 examples are shown in **Figure 3**). Neither did hydrolysis carried at 40 & 60 °C at pH 1. The results suggest that these conditions were insufficient to break the glycosidic linkages to any significant extent. In contrast, new bands were seen for the sample degraded for 24 h and 48 h at pH 1/80 °C (highlighted by the yellow box, **Figure 3**). With reference to an oligosaccharide (labelled dp 2-10) standard ladder, the weak new bands were located between dp 8 and dp 2 oligosaccharides, and their intensity increased with hydrolysis time. It can therefore be concluded that during prolonged acidic treatment (pH 1/ 80 °C/ from 24 h) heparin chains start to gradually degrade.

Figure 3. Illustrative acrylamide gels images of hydrolysed heparin, showing its stability to mild acidic conditions. Significant degradation was observed only for the strongest conditions (pH 1/ 80 °C, 24 & 48 h), highlighted in yellow. Std - standard, dp - degree of polymerization, H₂O – blank load.

3.3 Monosaccharide analysis

To further investigate potential heparin degradation, the hydrolysates were analysed with HPAEC-PAD. This quantitative analytical protocol has been developed based on previous studies, describing a single chromatographic method for the analysis of neutral and amino sugars together with uronic acids^{48,49}. The injection of an aqueous solution of heparin sodium API confirmed the initial purity of the compound in terms of the absence of free monosaccharides. The monosaccharide analysis of the sample hydrolysed in TFA at 100 °C for 24 h was used to mirror the total degradation profile of heparin i.e. as a positive control (an exemplar chromatogram is shown in Supporting Information - Figure 3). The presence of GlcN/GlcNS, GlcA and IdoA was based on the assignment of standards shown in Supporting Information - Figure 1. The remaining

peaks eluted between 20- 40 min, were most likely disaccharides and oligosaccharides of heparin. Their appearance matches the PAGE and TLC results (data not shown) and is consistent with previous results presented elsewhere⁴⁸.

The hydrolysates of heparin were analysed to profile the composition of the polymer exposed to acidic environments (pH 1 - 6) at three different temperatures (40, 60, 80 °C) as a function of time. The low levels of released GlcN/GlcNS and low levels of unidentified elements were observed only in samples degraded in pH 1 at 80 °C for 24 and 48 h. The detected peaks most probably mirror bands eluted on acrylamide gels (**Figure 3**), consistent with HPAEC-PAD results, while chromatographically detected glucosamine monosaccharides could diffuse out of the PAGE gels and evade detection. Since the structure of repeating disaccharide of heparin (**Figure 1**) is composed of glucosamine and uronic acid residues at a ratio of 1:1, the hydrolytic release should lead to detection of both units. Although the method was optimised with regards to uronic acids (see Supporting Information), the peaks of acidic sugars were not detected in any of the hydrolysed samples. This could be explained primarily by the partial degradation of heparin chain. Since the glycosidic linkages of uronic acids are highly resistant to acid hydrolysis,⁴⁰ the detected peaks are likely to be the result of minor, although preferential, cleavage of GlcN/GlcNS-(1→ bonds, that through a non- selective pathway creates mono- and/ or oligo- residues, that would result in elution profiles similar to those presented in **Figure 4**. On the other hand, considering the findings of another study, that suggested the limited stability of iduronic acid to prolonged acidic treatment,⁴⁸ it could be that initially released acidic residues would be subject to degradation. Given that the first bands/peaks (PAGE and HPAEC respectively) marking the beginning of chain degradation were observed after 24 hours of hydrolytic treatment in pH 1/ 80 °C, and subsequent analysis concerns sample aliquoted after another 24 h of continuous exposure to harsh acidic environments,

the uronic acids (especially IdoA) could degrade over this period of time. All things considered, the unknown peaks eluted from the pH 1/ 80 °C/ 48 h sample (**Figure 4b**), could equally represent heparin oligosaccharides and/or adducts of iduronic acid.

Figure 4. Illustrative elution chromatogram of heparin hydrolysed at **a)** pH 1/ 80 °C/ 24 h and **b)** pH 1/ 80 °C/ 48 h. The GlcN/GlcNS peak was assigned according to elution of calibration standards, shown in Supporting Information- Figure 1. The unidentified peaks, marked as *, likely represent heparin oligosaccharides and/or adducts of iduronic acids.

3.4 The influence of desulfation on molecular weight

Rate of desulfation, by analysis of sulfate ion release: Considering that (small amounts) of mono- and oligosaccharides (or adducts of iduronic acids) were detected only in samples hydrolysed under harsh conditions (pH 1/ 80 °C/ from 24 h), the origin of the molecular weight loss observed

at milder hydrolytic environments (as presented in **Figure 2**) remained unresolved. As previously demonstrated, sulfated polysaccharides, like heparin, have the potential to lose sulfate groups prior to depolymerisation.^{2,26,50} Thus, the presence of sulfate anions in post-hydrolytic samples was ascertained using HPAEC-CD. The analytical protocol was an adaptation of Liu's method (2014), taking advantage of ion chromatography.⁵¹ Details of method optimisation are given in Supporting Information.

Since the analysis of the heparin standard confirmed the absence of free sulfate in the starting material, the subsequent detection of sulfate anions confirmed hydrolytic desulfation. The free sulfate anions were detected in heparin samples hydrolysed under the following conditions: 40 °C: pH 1 - 2, 60 °C: pH 1 - 3 and 80 °C: pH 1 - 4. No anions were detected in the samples exposed to pH 5 or 6 at any temperature. As illustrated in **Figure 5**, the concentration of detected sulfate increased as a function of time and temperature in all cases. To be more specific, at 40 °C, the pH 1 sample exhibited a fairly consistent sulfate rise as a function of time, while at pH 2, the sulfate was only detected after 36 h of processing (**Figure 5a**). At 60 °C and 80 °C, the sulfate concentration increased rapidly during the first 12 h and the increase gradually slowed down until the reaction was terminated, except at pH 1, which featured an additional concentration rise ≥ 36 h (**Figure 5b-c**). The observed change of rate may be the result of ordered desulfation phenomena suggested by earlier studies focusing on heparin stability,^{28,29,32} and it was hypothesised as resulting from the loss of sulfate from different positions. This hypothesis formed the foundation for the following analysis, described in **section 3.5**.

Figure 5. Measured concentration of sulfate anions of thermally stressed heparin at (a) 40 °C, (b) 60 °C and (c) 80 °C plotted as a function of time at pH 1- 6. The vertical error bars represent standard deviation from the mean of concentration, calculated from peak area *via* calibration curve method (where not visible, the error bars are concealed by data points). • pH 1, • pH 2, • pH 3, • pH 4, • pH 5, • pH 6

Influence of desulfation on total molecular weight loss: The final concentrations of sulfate ($C_{sulfate}$), calculated for each hydrolytic sample at 48 h are summarized in **Table 1**. Considering that the concentration of sulfate anions detected in the pH 1/ 80 °C sample ($C_{sulfate} = 1092$ ppm), accounts for ~ 1/5 of total concentration of initial sample ($C_{initial} = 5000$ ppm), the effect of sulfate loss on the average weight molecular weight of heparin was questioned. Hence, the mass of sulfate ($M_{sulfate}$), was estimated as a fraction of initial molecular weight of polysaccharide ($M_{w,0}$ Hp ≈ 20,000 g/mol), according to equation 1:

$$M_{sulfate} = \bar{M}_{w,0} \text{ Hp} \left(\frac{g}{mol} \right) \times \frac{C_{sulfate} (ppm)}{C_{initial} (ppm)} \quad (1)$$

The calculated masses of hydrolytically released sulfate were further subtracted from the $M_{w,0}$ Hp, giving the theoretical average weight molecular weight of heparin at each hydrolytic time point. The theoretical M_w and measured M_w (SEC-MALS-RI) of selected hydrolytic samples are summarized in **Table 2**.

Table 1. Sulfate concentration measured in heparin samples at 48 hours of hydrolysis, at pH 1 - 6, at 40 °C, 60 °C and 80 °C.

Temperature (°C)	Sulfate Concentration (ppm)					
	pH 1	pH 2	pH 3	pH 4	pH 5	pH 6
40	210 ^a ± 18 ^b	59 ± 16	n.d. ^c	n.d.	n.d.	n.d.
60	671 ± 21	425 ± 26	98 ± 15	n.d.	n.d.	n.d.
80	1092 ± 28	685 ± 21	406 ± 20	63 ± 17	n.d.	n.d.

^a Concentration calculated by calibration curve using peak area; ^b Error calculated from standard deviation of triplicated reading; ^c n.d.= not detected

The values of theoretical M_w and measured M_w of heparin hydrolysates (**Table 2**) were compared using Wilcoxon matched-pairs signed-rank test. In this non-parametric statistical approach, the differences between each set of matched data were computed and ranked, while the difference medians were tested against zero. As the p values of paired data were large ($p > 0.05$, per pH, per temperature), it was concluded that overall medians did not vary, therefore the values of matched M_w pairs were not significantly different. Consequently, it was deduced that under the applied conditions, desulfation could account for the most of the observed decrease in molecular weight of heparin. Even though the PAGE and HPAEC-PAD studies (see **sections 3.2 & 3.3**) showed the presence of mono-/oligosaccharides in pH 1/ 80 °C/ 24 & 48 h, the glycosidic scission must have occurred to only a low extent. Ergo, in comparison to the loss following desulfation, the observed depolymerisation would not substantially influence the molecular weight of heparin. Moreover, the oligosaccharide positions on the PAGE gels (**Figure 3**) and their HPAEC-PAD retention times did not match precisely with the expected sulfated oligo-standards, suggesting that the residues could themselves be de-sulfated and/or arise from adducts of iduronic acid.

Table 2. The comparison of theoretical and measured average weight molecular weight of selected heparin hydrolysates.

Heparin Hydrolysates		Theoretical M_w (g/mol) ^a $\times 10^4$: Measured M_w (g/mol) $\times 10^4$		
		40 °C	60 °C	80 °C
pH 1	6 h	1.98 : 1.84	1.85 : 1.81	1.76 : 1.63
	12 h	1.96 : 1.77	1.79 : 1.75	1.71 : 1.62
	24 h	1.95 : 1.74	1.79 : 1.68	1.66 : 1.61
	48 h	1.92 : 1.65 ^b	1.73 : 1.60	1.56 : 1.46 ^c
		p ^d = 0.1250	p = 0.1250	p = 0.2500
pH 2	6 h	1.99 : 1.99	1.96 : 1.85	1.88 : 1.86
	12 h	1.99 : 1.98	1.92 : 1.79	1.81 : 1.78
	24 h	1.99 : 1.98	1.89 : 1.76	1.77 : 1.75
	48 h	1.98 : 1.79	1.83 : 1.70	1.73 : 1.55
		p = 0.1250	p = 0.1250	p = 0.1250
pH 3	6 h	1.99 : 1.99	1.99 : 1.91	1.95 : 1.82
	12 h	1.99 : 1.99	1.98 : 1.81	1.93 : 1.79
	24 h	1.99 : 1.99	1.98 : 1.80	1.90 : 1.79
	48 h	1.99 : 1.95	1.96 : 1.79	1.84 : 1.70
		p = 0.1250	p = 0.1250	p = 0.1250

^a Theoretical M_w (g/mol) calculated by subtracting the amount of released sulfate anions (g/mol), measured by HPAEC-CD, from the M_w of heparin sodium standard, measure by SEC-MALS-RI. ^{b, c} pairs of theoretical and measured that M_w (g/mol) and demonstrated not significantly effective pairing; ^d calculated value, if p > 0.05, compared data are not significantly different

3.5 NMR reveals unexpected order of hydrolysis

The anion analysis of the hydrolytic samples helped to establish that the reduction of molecular weight of heparin was primarily caused by hydrolytic sulfate loss, rather than chain scission. The change of desulfation rate (over time) suggested that the process involved different sulfate groups. According to earlier studies, the desulfation of heparin under mild acidic conditions targeted the N-sulfate first, followed by de-O-sulfation, which could be written schematically as $OS \ll NS$.^{28,29} Studies of heparin degradation in mild acidic solvolytic conditions allowed the expansion of the de-O-sulfation order, pointing out that in organic medium the 6-O-sulfate of glucosamine is removed prior, and with high selectivity, to 2-O-sulfate of iduronic acid.^{33–35} Thus, the order solvolytic de-sulfation could be written schematically as: $2OS < 6OS \ll NS$.

NMR spectroscopy offers an opportunity to follow any structural changes in the polymer chain by monitoring the chemical shifts of $^1H/^{13}C$ signals which have been assigned.^{47,52,53} Therefore, the method was used to examine heparin samples and assess how the hydrolytic de-sulfation observed in this study fits the previously assumed order of de-sulfation.

The HSQC heparin standard (Hp) spectra were compared with the spectra of samples hydrolysed at pH 1, aliquoted at 24 and 48 hours and processed at 40, 60 and 80 °C. To track any structural changes and to determine the desulfation order, the chemical shifts of these samples were compared. The data used for comparative analysis are summarized in **Table 3**, while exemplar NMR spectra (^{13}C) are illustrated in **Figure 6**.

Figure 6. One dimensional ^{13}C NMR spectra of heparin **standard** (50 mg/0.6 mL D_2O) and pH 1/ 48 h **a)** 40 °C, **b)** 60 °C and **c)** 80 °C hydrolysis products (~ 20 mg/0.6 mL D_2O). All spectra were recorded at 333K on a Bruker 600 MHz spectrometer with acetone (10 μL /100 mL D_2O) as the reference standard. Signals (**standard**) were assigned according to Yates *et al.* (1996). The peak shifts of the hydrolysed sample were confirmed with HSQC/ ^1H spectra (data not shown). A- glucosamine residues, I- iduronic acid residues.

Table 3. ^{13}C and ^1H chemical shift data for heparin standards and pH 1 heparin hydrolysates.

pH 1 aliquot		¹ H/ ¹³ C chemical shift (ppm)												
		Peak ^a	A-1	A-2	A-3	A-4	A-5	A-6	A-6	I-1	I-2	I-3	I-4	I-5
		I _{2S} -ANS(6X)	ANS(6X)	ANS(6X)	ANS(6S)	ANS(6S)	ANS(6S)	ANY(3X,6S) or ANX(6X)	I _{2S} -ANS(6X)	I _{2S}	I _{2S}	I _{2S}	I _{2S}	
Heparin standard		5.38 99.4	3.27 60.6	3.67 72.4	3.76 78.9	4.02 71.9	3.85 62.3^b	4.28-4.38 69.1	5.21 102.0	4.34 78.7	4.20 72.0	4.11 78.8	4.79 72.2	
de-N-sulfated standard		5.42 94.1	3.39 57.2	3.97 71.4	3.82 78.9	4.04 72.3	3.85 62.3	4.15-4.31 69.1	5.27 101.7	4.38 75.8	4.39 65.8	4.21 73.4	4.92 70.3	
40 °C	24 h	5.41 93.7	3.40 56.9	3.66 72.5	3.75 79.2	4.01 71.9	3.84 62.3	4.27-4.38 69.1	5.21 102.0	4.35 78.7	4.37 65.4	4.10 78.8	4.90 69.9	
		5.41 93.7	3.40 56.9	3.68 72.5	3.75 79.2	4.01 71.8	3.84 62.3	4.27 69.1	5.21 102.0	4.35 75.5	4.37 65.4	4.10 78.6	4.90 69.9	
	48 h	5.41 93.7	3.40 56.9	3.68 72.5	3.75 79.2	4.01 71.8	3.84 62.3	4.27 69.1	5.21 102.0	4.35 75.5	4.37 65.4	4.10 78.6	4.90 69.9	
		5.41 93.7	3.40 56.9	3.68 72.5	3.75 79.2	4.01 71.8	3.84 62.3	4.27 69.1	5.21 102.0	4.35 75.5	4.37 65.4	4.10 78.6	4.90 69.9	
60 °C	24 h	5.41 93.8	3.40 57.0	3.96 71.1	3.80 79.2	4.02 72.2	3.85 62.3	4.29 68.8	4.96 104.7	4.36 75.6	4.36 65.6	4.19 73.2	4.90 70.0	
		5.42 93.8	3.40 57.0	3.96 71.1	3.80 79.2	4.02 72.2	3.85 62.3	4.29 68.8	4.95 104.7	4.37 75.6	4.37 65.6	4.19 73.2	4.90 70.0	
	48 h	5.42 93.8	3.40 57.0	3.96 71.1	3.80 79.2	4.02 72.2	3.85 62.3	4.29 68.8	4.95 104.7	4.37 75.6	4.37 65.6	4.19 73.2	4.90 70.0	
		5.42 93.8	3.40 57.0	3.96 71.1	3.80 79.2	4.02 72.2	3.85 62.3	4.29 68.8	4.95 104.7	4.37 75.6	4.37 65.6	4.19 73.2	4.90 70.0	
80 °C	24 h	5.42 93.8	3.40 57.0	3.96 71.0	3.80 79.2	4.03 74.2	3.85 62.3^c	4.29 68.8^d	4.95 104.7	4.36 75.6	4.34 65.6	4.17 73.2	4.88 70.0	
		5.40 93.8	3.37 57.0	3.86 71.0	3.75 79.4	3.96 74.2	3.82 62.3	4.29 68.8	4.95 104.7	4.34 75.5	4.34 65.5	4.17 73.0	4.88 69.7	
	48 h	5.40 93.8	3.37 57.0	3.86 71.0	3.75 79.4	3.96 74.2	3.82 62.3	4.29 68.8	4.95 104.7	4.34 75.5	4.34 65.5	4.17 73.0	4.88 69.7	
		5.40 93.8	3.37 57.0	3.86 71.0	3.75 79.4	3.96 74.2	3.82 62.3	4.29 68.8	4.95 104.7	4.34 75.5	4.34 65.5	4.17 73.0	4.88 69.7	

^a number next to monomer signal mark the carbon number; ^b position from HSQC spectrum, ^{13}C not visible in 1D NMR; ^c signal visible in 1D and 2D spectra, due to 6-de-O-sulfation at ^dA-6; A - glucosamine; I - iduronic acid; N - Nth proton/ carbon of glucosamine residue; S- sulfated; X= H or -SO₃-; Y= H or Ac or -SO₃-. various colours mark most prominent changes in **40 °C**, **60 °C** and **80 °C** NMR spectra

Loss of heparin N-sulfate groups at 40 °C demonstrates the priority of N-desulfation: The removal of an N-sulfate group from the polymeric backbone causes the upfield shift at the carbon from which the sulfate group is detached (C-2 of the glucosamine residue), by lowering the deshielding effect. This modification also causes the I-3 signal to resonate further upfield, as a consequence of the interaction between the hydroxyl at position-3 of IdoA and the N-sulfate, which has been noted previously.⁵² When comparing standard heparin with the pH 1/ 40 °C/ 24 h sample, the partial upfield shifts from 72.0 to 65.4 ppm (Δ = 6.6 ppm) and from 72.2 to 69.9 ppm (Δ = 2.3 ppm) in ^{13}C spectrum, as well as downfield shifts from 4.20 to 4.37 ppm (Δ = 0.17 ppm) and from 4.79 to 4.90 (Δ = 0.11 ppm) in ^1H spectrum, were noted respectively at positions I-3 and I-5 (**Table 3**). In the spectrum of the pH 1/ 40 °C/ 48 h sample, the additional upfield shifts, *i.e.*: 99.4→ 93.7 ppm (Δ = 5.7 ppm), 60.6→ 56.9 ppm (Δ = 3.7 ppm) and 78.7→ 75.5 ppm (Δ = 3.2 ppm), were observed at the positions A-1, A-2 and I-2, respectively in ^{13}C (**Figure 6a**). While the ^1H positions of these peaks changed by 0.03, 0.13 and 0.01 ppm, downfield (**Table 3**). The observation of these shifts under relatively low temperatures confirms the susceptibility of heparin to initial de-N-sulfation, noted in earlier studies discussing its hydrolytic degradation.^{28,29,32}

Unexpected, further desulfation preferences revealed: The spectra analysis of the pH 1/ 60 °C samples showed that A-1, A-2, I-2, I-3 and I-5 signals were completely shifted (to the positions discussed in the paragraph above) after 24 h of processing. Considering the ‘shift termination’ of peaks that were earlier associated with de-N-sulfation (at 40 °C), the pH 1/ 60 °C spectra were checked against a de-N-sulfated heparin standard, as shown in Figure 5- Supporting Information. The match between the $^1\text{H}/ ^{13}\text{C}$ positions of the glucosamine and iduronate peaks suggested that, after 24 h of hydrolytic degradation at pH 1/ 60 °C, the heparin molecule was completely de-N-sulfated.⁵²

The analysis of pH 1/ 60 °C revealed another interesting phenomenon. Namely, the ^{13}C signal of I-1 peak clearly started moving downfield, from 102.0 to 104.7 ppm ($\Delta = 2.7$ ppm), while the ^1H signal shifted upfield, from 5.21 to 4.96 ppm ($\Delta = 0.25$ ppm) (**Table 3 & Figure 6b**). This gradual change has been perfectly captured in HSQC spectra of pH 1/ 60 °C/ 48 h sample, presented in **Figure 7**. Following the study of Yates *et al.* (1996), which discussed the changes of chemical shifts of heparin peaks in reference to different sulfation patterns,⁵² the displacement of I-1 peak evidently marks the beginning of de-sulfation at the position 2-O- of iduronate. At the same time, as observed in **Figure 7**, the $^1\text{H}/^{13}\text{C}$ chemical shifts of signal A-6 remained unaltered (also see **Table 3**). Considering the ongoing shift of peak I-1 in the absence of changes at position A-6, it could be concluded, that under the applied hydrolytic conditions, 2-de-O-sulfation is, in fact, in preference to 6-de-O-sulfation. To highlight the importance of this finding, it should be recapped that until now, the hydrolytic desulfation of heparin was generalised as OS<<NS,^{28,29} which identifies the selective N-sulfate loss with subsequent O-sulfate scission. Whereas the O-sulfate scission was thought to follow the de-sulfation mechanism of solvolytic environments, *i.e.* prior 6-O-sulfate loss with consecutive 2-de-O-sulfation.^{33–35} While in fact, according to the detailed NMR analysis presented here, the hydrolytic de-O-sulfation seems to favour 2-O-sulfate over the 6-O-sulfate.

Figure 7. The NMR spectra (HSQC) of heparin hydrolysed at pH 1/ 60 °C/ 48 h (orange) over the standard sample (black). The green arrows and grey region mark the shift of peaks in a) anomeric and b) aliphatic regions. The shift of A-1 and A-2 marks completed N-desulfation, while changes of I-I reflect 2-O-desulfation.

The final piece in the de-sulfation puzzle: To finalise the hydrolytic desulfation study of heparin, the sample hydrolysed at pH 1/ 80 °C was analysed from the 6-de-O-sulfation perspective. The previous studies confirmed that loss of 6-O-sulfate is associated with the major chemical shift of A-6 and A-5 carbon signals.^{47,52} The ^{13}C spectra comparison of heparin standard and pH 1/ 80 °C, indeed, revealed the gradual upfield changes from 69.1 to 62.3 ppm (6.9 ppm) at positions A-6 and downfield move of the A-5 signal, from 71.9 to 74.2 ppm (2.3 ppm). The proton positions of

both peaks moved upfield by 0.03 and 0.06 ppm, (**Table 3**). The displacement of these peaks can be seen more clearly in HSQC spectrum, presented in **Figure 8**. At the same time, the progressive movement of the I-1 signal (102.0→ 104.7 ppm), marking the continuing 2-O-desulfation, was also observed (**Figure 8 & Table 3**). This suggests that the acid-catalysed, hydrolytic 6-de-O-desulfation is not conditional upon 2-O-sulfate loss, and therefore these two processes could be considered as independent of each other.

To get more perspective on the hydrolytic de-O-sulfation selectivity, the approximate integrals ratio of signals I-1 and A-6 were studied. Assuming the complete de-N-sulfation of glucosamine residues, at pH 1/ 60 °C/ 24 h, the approximate integrals ratio of I-1 signals, *i.e.* $I_{2S} : I_{OH}$, was 6.7:1, while the ratio of integrated A-6 signals, *i.e.* $A_{6S} : A_{OH}$, was 4.1:1. As the $A_{6S} : A_{OH} < I_{2S} : I_{OH}$, it could be concluded that under hydrolytic conditions of pH 1/ 60 °C, there is a preference towards secondary sulfate (2-O-S-) loss over the 6-O-sulfate. On the other hand, at the last set of studied environments, *i.e.* pH 1/ 80 °C (also 24 h), the same comparison shows that the 6-O-sulfate exhibits 1.5-fold greater hydrolytic potential than previously. As both integral ratios of I-1 and A-6 signals increased together with applied conditions, and as the 6-de-O-sulfation escalation was more dominant, the ‘selectivity’ assumptions of hydrolytic de-O-sulfation (either 2S- or 6S-) would appear to be inadequate under these conditions. Instead, it was clearly highlighted that the acid-catalysed hydrolytic desulfation of heparin is strongly dependent upon the applied conditions, starting with N-sulfate loss, with following 2-de-O-sulfation, while 6-O-sulfate is targeted last. Thus, the hydrolytic degradation/desulfation of heparin should certainly not be considered to follow the same order as solvolysis. Furthermore, once optimised, the hydrolytic conditions could show potential toward ‘more selective’ de-O-sulfation, perhaps complementary to the solvolytic reaction.

In addition, a new peak was observed at 5.48 ppm/ ^1H ; 94.8 ppm/ ^{13}C , as marked in **Figure 8**. Bearing in mind that these conditions are sufficient to initiate glycosidic scission (see **sections 3.2-3.3**), the signal may represent C-1 of free glucosamine.^{46,54} This was the ‘only’ new signal characterised in the NMR spectrum of post-hydrolysed sample, and matches the monosaccharide eluted during HPAEC-PAD analysis (see **section 3.3**). Although it would be challenging to distinguish the low levels of oligosaccharides eluted in HPAEC-PAD analysis, since their NMR spectra probably resemble the original heparin spectrum,⁵⁵ the NMR analysis complements the hypothesis of partial degradation of the molecule (**section 3.3.**) and suggests that primarily only one terminal glucosamine residue is removed from a heparin chain.

Figure 8. The NMR spectra (HSQC) of heparin hydrolysed at pH 1/ 80 °C/ 48 h (red) over the standard sample (black). The green arrows and grey region mark the shift of peaks in **a)** anomeric and **b)** aliphatic regions. The shift of A-6 marks the beginning of 6-O-desulfation, while I-I reflect continuous 2-O-desulfation. The new peak signed and circled in **a)** probably reflects C-1 of severed glucosamine unit.

3.6 Updated mechanism for the hydrolytic degradation of heparin in acidic conditions

The NMR analysis discussed in section 3.5, has allowed us to complement the hydrolytic desulfation order of heparin, proposed by Danishefsky (1960) and Inoue/Nagasawa (1975),^{28,29} and further its applicability, under the conditions studied here, to the following order: de-N-

sulfation, being the most prominent and selective process, followed by 2-de-O-sulfation and finally 6-de-O-sulfation of lesser selectivity, that schematically could be written as $6OS < 2OS < NS$.

This unexpected observation required us to revisit the previously proposed mechanism of acid-catalysed, hydrolytic degradation of heparin. Moreover, considering the significant influence sulfate (loss) has on the total molecular weight of the polymer, the desulfation process should certainly be included as the initial degradative step. Hence, in **Figure 9**, we propose a refinement to the mechanism previously suggested by Jandik (1996),³² highlighting the successively removed sulfate groups in yellow.

Our study establishes the hydrolytic desulfation order under aqueous acidic conditions, however, it does not necessary explain why the degradation follows this sequence. One possibility is that the use of ionic aqueous solutions may alter hydrogen bonding networks and/or conformation. This may result in an ionic interaction between the protonated amine of the glucosamine and the negative sulfate of the IdoA that alters the conformation of the IdoA residue, introducing strain into its (C2)-O-S bond and/or altering the polarisation of the bond making it easier to break. One should recall the literature justifying the rate of sulfate cleavage by their location on the individual sugar residue and general sensitivity ($NS < OS$) toward the acidic scission.^{29,33,56} A more in-depth study, considering the detailed kinetics and rearrangements of the particular sulfates groups, is potential future work.

Figure 9. Proposed mechanism of hydrolytic degradation of heparin in acidic environments based on the conclusions drawn from the experimental study presented in this article. The individual sulfate groups, liberated at each characteristic stage of mechanism are highlighted in yellow. The last stage of mechanism assumes two pathways: one targeting the GlcN/GlcNS-(1 $\rightarrow$ bond and resulting in GlcN monomer and heparin oligomers (left), and one that assumes the scission of IdoA and its further degradation (right). The bracket left after the final arrow indicates the (probable) subsequent degradation of the polymer chain. R= H or SO_3^- (conditional upon applied factors).

CONCLUSIONS

Through a variety of analytical techniques, the acid hydrolysis of heparin has been followed at 40, 60 and 80 °C. After connecting all the pieces from the complex analytical puzzle, a more complete understanding of the degradation mechanism was elucidated.

As expected, heparin appeared to be resistant to glycosidic scission at pH values (pH 1 - 6), both at 40 °C and 60 °C. However, under these conditions, the amount of hydrolytically cleaved sulfate could account for the polymer molecular weight loss. This was also the case for samples hydrolysed at pH 2 - 6, at 80 °C. Minor glycosidic scission was observed after the degradation of polymer for 24 h at pH 1, at 80 °C. The NMR analysis of post-hydrolytic samples provided insight into macromolecular changes and revealed an unexpected order of desulfation. In contrast to solvolysis, the initial desulfation preferences of hydrolytically degraded heparin at pH 1/ ≤60 °C/ ≤48 h, can be approximated by; 6OS < 2OS << NS, although higher temperature (80 °C), associated with glycosidic chain scission, reduces the selectivity of de 2-O- over de 6-O-sulfation. Furthermore, the monosaccharide analysis combined with NMR results suggest partial chain degradation and release of terminal glucosamine units. Finally, the desulfation order and glucosamine scission were included in a refined, general acid-catalysed hydrolytic degradation mechanism of the molecule. The present analytical approach, especially NMR screening of the hydrolysed samples, has potential in studies of the stability of complex macromolecules.

SUPPORTING INFORMATION

Detailed description of chromatographic methods; elution chromatograms of standards; statistical analysis of chromatographic standards and calibration curves of chromatographic standards.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. AMK was responsible for the methodology, investigation, data interpretation and analysis and the writing of the original draft; EAY provided the ANDSA tagged-heparin oligosaccharides, heparin standards and was responsible for methodology, data interpretation and analysis, writing - review & editing. KT, JR, AMS and GAM were responsible for the conceptualization, resources, data interpretation and analysis, writing - review & editing and supervision.

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