Identification of a novel structure in heparin generated by potassium permanganate oxidation

Daniela Beccati [a,1], Sucharita Roy [a,1], Fei Yu [a], Nur Sibel Gunay [a], Ishan Capila [a], Miroslaw Lech [a], Robert J. Linhardt [b], Ganesh Venkataraman [a,*]

[a] Momenta Pharmaceuticals, 675 West Kendall Street, Cambridge, MA 02142, USA
[b] Departments of Chemistry and Chemical Biology, Biology, and Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Center for Biotechnology and Interdisciplinary Studies, 110 8th Street, Troy, NY 12180, USA

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ABSTRACT

The worldwide heparin contamination crisis in 2008 led health authorities to take fundamental steps to better control heparin manufacture, including implementing appropriate analytical and bio-analytical methods to ensure production and release of high quality heparin sodium product. Consequently, there is an increased interest in the identification and structural elucidation of unusually modified structures that may be present in heparin. Our study focuses on the structural elucidation of species that give rise to a signal observed at 2.10 ppm in the *N*-acetyl region of the ^{1}H NMR spectrum of some pharmaceutical grade heparin preparations. Structural elucidation experiments were carried out using homonuclear (COSY, TOSCY and NOESY) and heteronuclear (HSQC, HSQC-DEPT, HMQC-COSY, HSQC-TOCSY, and HMBC) 2D NMR spectroscopy on both heparin as well as heparin-like model compounds. Our results identify a novel type of oxidative modification of the heparin chain that results from a specific step in the manufacturing process used to prepare heparin.

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1. Introduction

Heparin is a polydisperse, highly sulfated, linear polysaccharide comprised of repeating α1 → 4 linked pyranosyluronic acid and 2-amino-2-deoxyglucopyranose (D-glucosamine) residues. Within the heparin chain, the amino group of the glucosamine residue can be substituted with either an acetyl or a sulfo group. Additionally, the 3-*O* and 6-*O* positions of the glucosamine residues can either be non-sulfated or substituted with a sulfo group. Finally, the uronic acid of each disaccharide repeat unit can either be L-iduronic or D-glucuronic acid, and may contain a 2-*O* sulfo group (Gunay & Linhardt, 1999; Linhardt, 2003; Sugahara & Kitagawa, 2002).

Heparin's primary therapeutic application is as an anticoagulant for the treatment and prevention of thrombotic disorders (Lindahl, 2000; Petitou, Casu, & Lindahl, 2003). Heparin treatment is usually well tolerated by patients. However, in late 2007 and early 2008, patients undergoing hemodialysis and receiving pharmaceutical heparin presented with severe adverse reactions, including angioedema, hypotension, and swelling of the larynx, which in

some cases ended in death. Proton nuclear magnetic resonance (^{1}H NMR) and capillary electrophoresis (CE) analysis of the administered lots revealed the presence of a contaminant which, after structural characterization, was identified as an oversulfated form of chondroitin sulfate (OSCS) (Guerrini et al., 2008). Biological studies confirmed that the presence and amount of OSCS correlated with activation of the contact system, leading to an anaphylactoid response (Kishimoto et al., 2008).

After the identification of the contaminant as OSCS, the regulatory authorities required heparin samples to be submitted to ^{1}H NMR and CE analysis to determine sample purity. Concomitantly, various compendia standard-setting organizations across the globe, among them the United States Pharmacopeia and the European Pharmacopeia, proposed implementation of defined analytical and bio-analytical methods to test properties and purity of Heparin Sodium. One key test within the revised monographs is ^{1}H NMR, which establishes regions within the spectrum of a heparin sample that must be free of any unidentified signal above a defined threshold.

Upon a survey of heparin samples using ^{1}H NMR analysis, we found that many samples (including the European Directorate for the Quality of Medicines heparin reference standard) presented an unidentified signal at 2.10 ppm, which might result from either an impurity or a modified structure within the heparin chain. In this

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Table 1
Description of samples used in this study.

Sample ID	Sample description
UFH lot 1	Unfractionated heparin lot that shows a signal at 2.10 ppm
UFH lot 2	Unfractionated heparin lot without a signal at 2.10 ppm
PI-HS	Porcine intestine mucosal heparan sulfate
PI-HS$_{ox}$	Porcine intestine mucosal heparan sulfate oxidized with potassium permanganate
PI-HSNAc	Porcine intestine mucosal heparan sulfate with a greater proportion of N-acetylglucosamine at the reducing end.
PI-HSNAc$_{ox}$	Potassium permanganate oxidation of porcine intestine mucosal heparan sulfate with a greater proportion of N-acetylglucosamine at the reducing end.

study, we demonstrate that the signal observed at 2.10 ppm is due to heparin related-structures generated during an oxidative step used in heparin purification. Treatment of unfractionated heparin and a heparin-like model compound with potassium permanganate showed that certain N-acetylglucosamine residues are easily oxidized, and the structural changes result in the chemical shift of N-acetyl protons to 2.10 ppm. Furthermore, our results identify the location and the potential structure(s) of the newly generated residue.

Finally, our research strategy also provides a general roadmap for the identification and structural elucidation of other unusually modified structures that may be present within heparin or other related polysaccharides.

2. Materials and methods

2.1. Materials

Unfractionated Heparin samples (UFH, porcine intestinal) were obtained from various heparin suppliers. Porcine intestinal heparan sulfate (PI-HS) was obtained from Celsus Laboratories (Cincinnati, OH, USA). Potassium permanganate (99%), hydrogen peroxide (30% (w/w) solution), Celite® and 3-(trimethylsilyl) propionic acid-d$_4$ sodium salt (TSP) were obtained from Sigma–Aldrich® (St. Louis, MO, USA). All reaction solutions were prepared using ultrapure water generated from a Sartorius ultra filtration unit. All other reagents were analytical grade. Deuterium oxide (D$_2$O, 99.9% D) and sodium deuteroxide (NaOD, 99.5% D) was purchased from

Cambridge Isotope laboratories, Inc. (Andover, MA, USA). Sodium borodeuteride (98% D) was obtained from Isotec (Miamisburg, OH, USA).

2.2. General procedure for oxidation using hydrogen peroxide

Briefly, the heparin sample at a 10 mg/mL concentration in ultra-pure water was adjusted to pH 11 and incubated with varying amounts of hydrogen peroxide (7–15% v/v) for 18 h at 30 °C. The pH and temperature of the reaction mixture was maintained through the course of the reaction. Upon completion, the pH of the reaction mixture was readjusted to neutrality and the reaction products isolated by salt/methanol precipitation. The products were obtained in 75–80% yields and further identified and characterized by NMR analysis.

2.3. General procedure for oxidation using potassium permanganate

Heparin, or model compounds, at a concentration of 10 mg/mL in ultrapure water were adjusted to pH 8.5–9.0 and incubated with varying amounts of potassium permanganate (2–24% w/w) for 2 h at 80 °C. The pH and temperature of the reaction solutions were monitored and maintained throughout the course of the reaction. Upon completion, the colored reaction solutions obtained were filtered through Celite® and the pH of the filtrate was adjusted to neutrality before isolation using salt/methanol precipitation. The products were obtained in 50–70% yields and were further identified and characterized by NMR analysis.

2.4. NMR analysis

Samples for NMR analysis were dissolved at 15–30 mg/0.7 mL of D$_2$O (99.9%) and sonicated for 30 s to remove air bubbles. Spectra were recorded at 298 K using either a 600 MHz Varian VNMRS spectrometer or a 600 MHz Bruker Avance 600 spectrometer, both equipped with a 5-mm triple-resonance inverse cryoprobe. ^{1}H NMR spectra were acquired with presaturation of the residual water signal, with a recycle delay of 7 s, for 24 scans. COSY spectra were recorded with presaturation of the water signal, for 24 scans of 256 increments. TOCSY spectra were acquired in phase sensitive mode with 80 ms of DIPSI-2 mixing, for 32 scans of 256 increments. For COSY and TOCSY spectra, the matrix size was zero filled to 2 × 2 K prior to Fourier transformation. HSQC and HSQC-DEPT spectra were recorded with sensitivity enhancement and carbon decoupling during acquisition, for 12–96 scans of 320 increments. The polarization transfer delay was set with a $^1J_{C-H}$ coupling value of 155 Hz. For HSQC spectra, the matrix was zero filled to 2 × 1 K
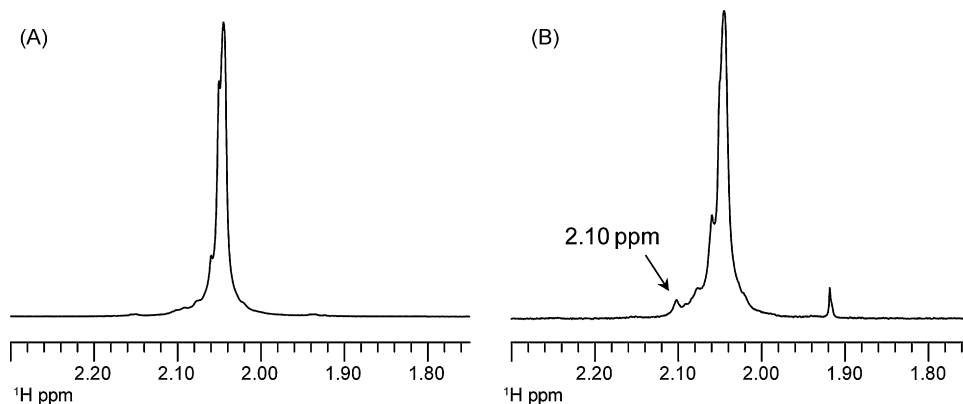

Fig. 1. ^{1}H NMR spectra of (A) unfractionated heparin lot 2. The signal at 2.10 ppm is absent. (B) Unfractionated heparin lot 1. The signal at 2.10 ppm is labeled. The signal at 1.92 ppm is due to acetate salt.
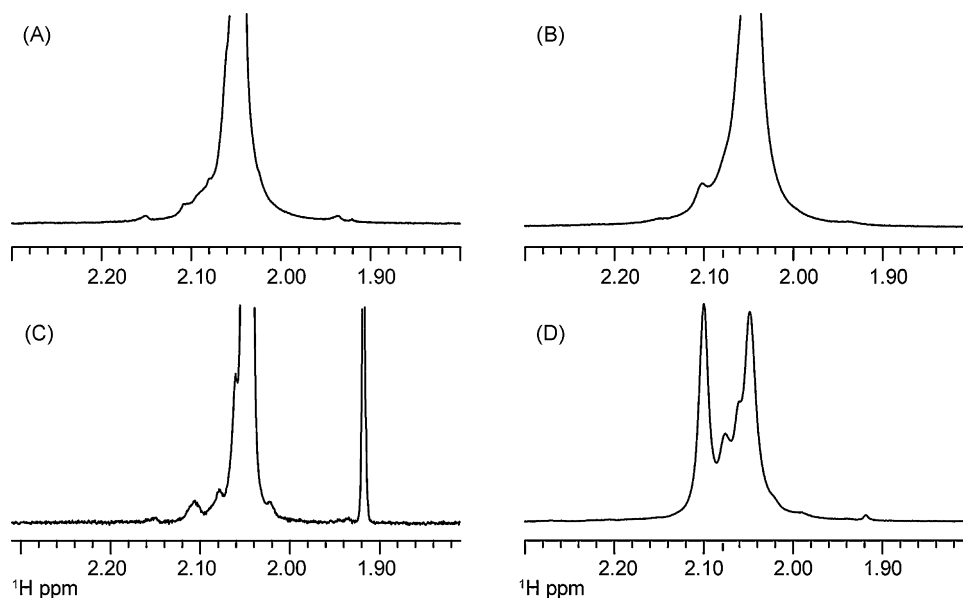
Fig. 2. ^{1}H NMR spectra of (A) PI-HS, (B) PI-HS$_{ox}$, (C) PI-HSNAc and (D) PI-HSNAc$_{ox}$. Only the *N*-acetyl region of the spectra is shown. The signal at 1.92 ppm is due to acetate salt.

prior to Fourier transformation. The HMBC spectrum was obtained without carbon decoupling and with a two-fold low-pass J-filter to suppress one-bond correlations, with 1600 scans for 256 increments. The delay for evolution of long-range couplings was set with a J_{lr} of 8 Hz. HMQC-COSY spectra were recorded with carbon decoupling during acquisition, for 16 scans of 256 increments. HSQC-TOCSY spectra were recorded with carbon decoupling during acquisition, 90 ms or 20 ms of MLEV17 mixing, for 396 scans of 160 increments. Chemical shift values were measured downfield from 3-(trimethylsilyl) propionic acid-d_4 sodium salt (TSP) as an external standard at 298 K.

To observe the amide proton signals, samples were dissolved at 20 mg/0.7 mL of D$_2$O:water 1:9 (v/v). Spectra were acquired at pH 6.0 and 298 K, using either a 600 MHz Varian VNMRS spectrometer or a 600 MHz Bruker Avance 600 spectrometer. COSY spectra were recorded with Watergate solvent suppression, for 16 scans of 128 increments. NOESY spectra were recorded with Watergate solvent suppression and 400 ms mixing time, for 64 scans of 128 increments.

3. Results

Pharmaceutical grade heparin samples from different suppliers were analyzed by ^{1}H NMR. Spectra showed that the signal at 2.10 ppm was present to varying amounts in some heparin lots and not observed in others (Table 1 and Fig. 1).

A heparin sodium sample which showed a signal at 2.10 ppm (UFH lot 1) was treated with increasing concentrations of deuterated sodium hydroxide (0.1 M, 0.2 M, and 0.5 M NaOD) at room temperature for 4 h to determine the stability of the 2.10 ppm signal to hydrolyzing conditions. The resulting samples were neutralized and analyzed by ^{1}H NMR. While the signal at 2.10 ppm was unaffected when the sample was treated with 0.1 M and 0.2 M NaOD, it disappeared upon treatment with 0.5 M NaOD (Supplementary Fig. 1). At this concentration of NaOD, we also observed a decrease in the intensity of the heparin *N*-acetyl signals at 2.04 ppm. Previous publications (Lewis, Nizet, & Varki, 2004) indicate that *O*-acetyl groups are labile to mild base treatment (e.g., 0.1 M NaOH) while *N*-acetyl groups require stronger hydrolyzing conditions. The signal at 2.10 ppm was therefore ascribed to be related to an *N*-acetyl group.

Next, we decided to evaluate conditions that would give rise to the signal at 2.10 ppm. A heparin sample lacking the signal at 2.10 ppm (UFH lot 2) was subjected to oxidation with different concentrations of hydrogen peroxide as described in the methods section. ^{1}H NMR spectra on the resulting samples did not show detectable signal at 2.10 ppm (Supplementary Fig. 2A). When the same starting material was oxidized with 2% potassium permanganate, NMR analysis of the resulting sample confirmed the appearance of a signal at 2.10 ppm (Supplementary Fig. 2B). However, in this case, the intensity of the 2.10 ppm signal did not increase significantly upon treatment with higher potassium permanganate concentrations (8% and 16% w/w), as indicated by integration of the ^{1}H NMR spectra (data not shown). To provide additional structural information, 2D-NMR (HSQC) experiments were performed on the heparin samples treated with 2%, 8% and 16% w/w potassium permanganate. The spectrum of the samples treated with 2% and 8% potassium permanganate showed intense signals belonging to the linkage region (anomeric cross peaks were observed at 4.66/106.7, 4.45/105.8, and 4.53/104.2 ppm), but these signals were absent for the samples treated with 16% permanganate concentrations. Furthermore, the degradation of the linkage region observed upon treatment of heparin with 16% potassium permanganate did not appear to cause a significant increase in the intensity of the signal at 2.10 ppm. These results suggest that most likely the signal at 2.10 ppm does not arise due to degradation of the linkage region.

To further examine the origin of this signal, we decided to use porcine intestinal heparan sulfate (PI-HS) as a model compound. PI-HS contains a higher percentage of *N*-acetylglucosamine as compared to heparin. A sample of porcine intestinal heparan sulfate (PI-HS) was subjected to potassium permanganate oxidation (8% w/w, Table 1). Analysis of the oxidized sample (PI-HS$_{ox}$) confirmed the appearance of a small signal at 2.10 ppm (Fig. 2B) comparable in intensity to the one observed in heparin samples treated (Supplementary Fig. 2B) with the same oxidizing conditions.

In conjunction with the above studies, we also generated a sample of porcine intestinal heparan sulfate enriched in reducing end *N*-acetylglucosamine residues (PI-HSNAc) and characterized this sample by NMR. The presence of reducing α and β *N*-acetylglucosamine residues was confirmed by multidimensional
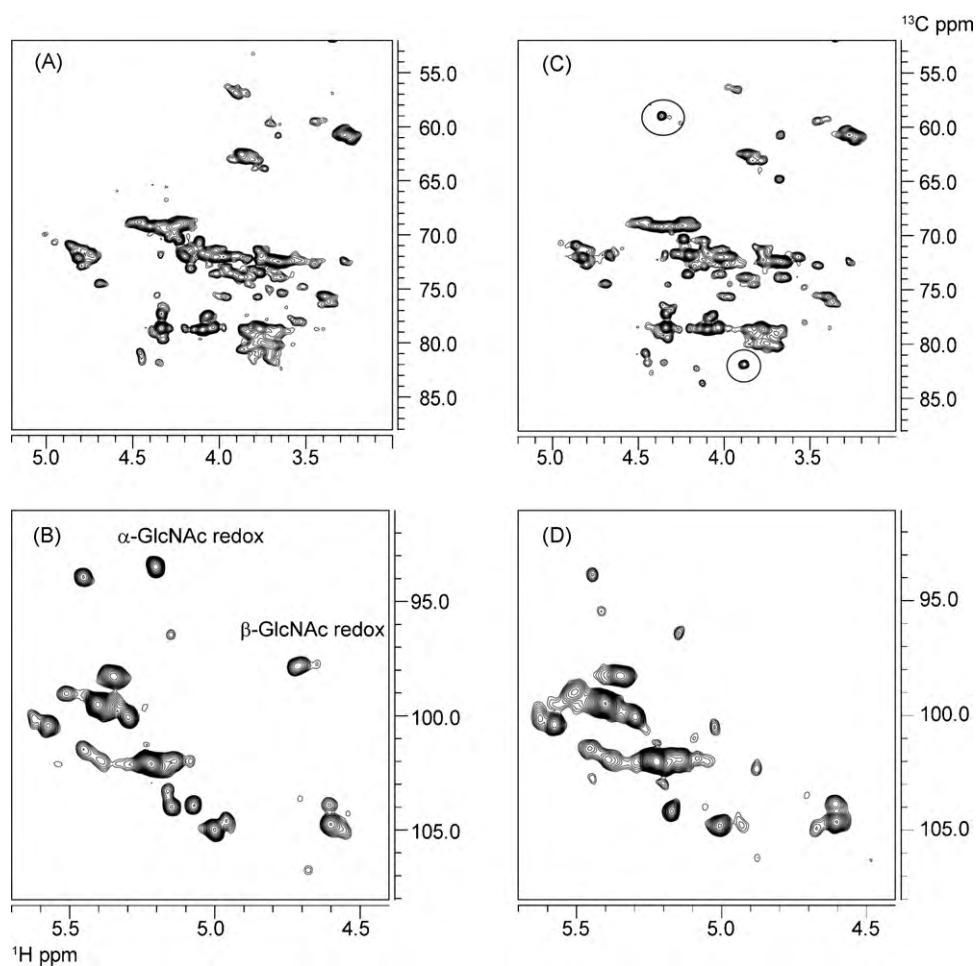
Fig. 3. HSQC spectrum of PI-HSNAc: (A) non-anomeric region and (B) anomeric region. Signals due to H1/C1 GlcNAc α and β reducing ends, are indicated. HSQC spectrum of PI-HSNAc$_{ox}$: (C) non-anomeric region (the cross peaks at 4.37/58.9 ppm and 3.88/81.8 ppm are circled), and (D) anomeric region.

experiments, i.e. COSY, TOCSY, and HSQC. These assignments were also consistent with literature data (Guerrini et al., 2007). The PI-HSNAc sample was subsequently subjected to potassium permanganate oxidation to generate PI-HSNAc$_{ox}$. The ^{1}H NMR spectrum of PI-HSNAc$_{ox}$ (Fig. 2D) showed an intense signal at 2.10 ppm, indicating that the oxidative chemistry had a significant influence on the N-acetylglucosamine residues at the reducing position of the heparin chain. To further evaluate the structural changes in these samples, both PI-HSNAc and PI-HSNAc$_{ox}$ were subjected to multidimensional NMR analysis (Fig. 3). Comparison between HSQC spectra of PI-HSNAc (Fig. 3B) and PI-HSNAc$_{ox}$ (Fig. 3D) demonstrated that signals due to reducing α and β anomeric signals of the reducing end N-acetylglucosamine residues disappeared after potassium permanganate treatment, while peaks due to internal N-acetylglucosamine (5.4–5.3/100.3–98.8 ppm) did not decrease in intensity.

Concomitantly, two distinct cross peaks appear at 4.37/58.9 ppm and 3.88/81.8 ppm (Fig. 3C) after potassium permanganate oxidation. An HSQC-DEPT experiment assigned the peak at 4.37/58.9 ppm to a –CH residue (Fig. 4A). A COSY experiment recorded in 10% deuterated water showed a cross peak between an amide proton (from the N-acetyl group) at 7.99 ppm and the peak at 4.37 ppm, while a NOESY experiment acquired in 10% deuterated water correlated the amide proton at 7.99 ppm to the CH$_3$ signal at 2.10 ppm. These data suggest that the peak at 4.37 ppm arises due to the H2 of the oxidized N-acetylglucosamine residue. In addition, HMBC analysis showed a long-range correlation between the proton at 4.37/58.9 ppm and

a carbonyl group (Fig. 4B). To determine if the carbonyl group belonged either to an aldehydic or carboxylic moiety, two experiments were performed. Firstly, the sample was acidified to pH 4.1 and an HSQC experiment was recorded. The experiment showed that the cross peak at 4.37/58.9 ppm shifted to 4.47/58.3 ppm as a function of the pH of the solution, consistent with a CH group adjacent to a carboxylic acid moiety. In addition, PI-HSNAc$_{ox}$ was treated with sodium borodeuteride (10%, w/w) for 60 min at 4 °C and, after neutralization, was analyzed by ^{1}H NMR (Supplementary Fig. 3). The intensity of the signal at 2.10 ppm did not decrease after reduction, indicating that the methyl group of the oxidized N-acetylglucosamine residue is adjacent to a carboxylic acid group. Furthermore, COSY and TOCSY experiments do not show any correlation of this peak (4.37 ppm) with signals present in the anomeric region, indicating that C1 does not have a corresponding proton. This observation supports the assignment of the C1 as a carboxylic acid moiety.

HSQC-TOCSY experiments show additional correlations between the peak at 4.37/58.9 ppm and signals at 4.21/73.6 ppm, 3.88/81.8 ppm, and 4.12/72.8 ppm (Supplementary Fig. 4). Assignment of these cross peaks were performed by analysis of HMQC-COSY and HSQC-TOCSY experiments recorded with different mixing times, and are reported in Table 2 and in Fig. 5. Additional cross peaks at 3.80/64.4 ppm and 3.72/64.4 ppm were also observed in the HSQC spectra of all the samples treated with potassium permanganate. HSQC-DEPT spectra indicate that these signals arise from CH$_2$ moieties (Fig. 4A). Correlations between the peaks at 3.80 and 3.72 ppm and other protons of
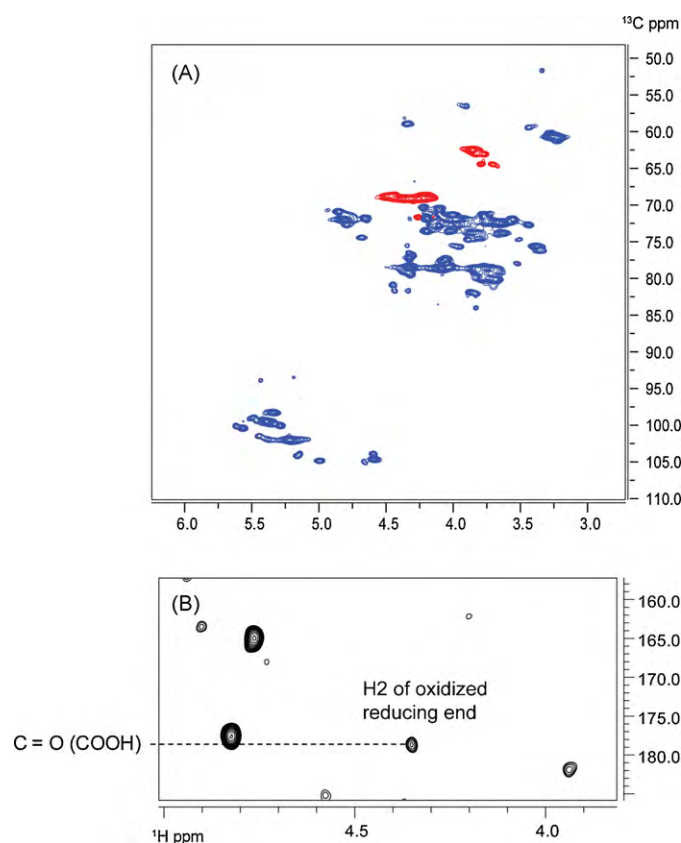
Fig. 4. (A) HSQC-DEPT spectrum of PI-HSNAc$_{ox}$. CH cross peaks are indicated in blue, CH$_2$ cross peaks are indicated in red. The cross peak at 4.37/58.9 ppm is circled. (B) HMBC spectrum of PI-HSNAc$_{ox}$. The long-range correlation between the proton at 4.37 ppm and a carbonyl group is indicated.

the oxidized residue could not clearly be identified by COSY and TOCSY experiments due to severe overlapping with other heparin signals. However, the proximity of the HSQC peaks at 3.80/64.4 ppm and 3.72/64.4 ppm to the H6,6′/C6 of 6-O-desulfated N-acetylglucosamine residues, and the appearance of these signals upon treatment with potassium permanganate, suggests assignment of these peaks to H6,6′/C6 of 6-O-desulfated N-acetylglucosaminic acid. The chemical shift assignments of the residue generated by potassium permanganate treatment are consistent with a 4-substituted N-acetylglucosaminic acid (Uchiyama, Dobashi, Ohkouchi, & Nagasawa, 1990).

Mass spectrometry was applied as an orthogonal analytical technique to support the structural assignment. The mass difference between N-acetylglucosamine and N-acetylglucosaminic acid at the reducing end is expected to be +16 Da. The PI-HSNAc$_{ox}$ sample was digested with Heparinase I and analyzed by gel permeation chromatography, followed by mass spectrometry. The

Table 2
NMR assignment of the oxidized reducing end (N-acetylglucosaminic acid).

	Heparin/Heparan sulfate	
	^{1}H	^{13}C
C1	–	178.8
H2/C2	4.37	58.9
H3/C3	4.21	73.6
H4/C4	3.88	81.8
H5/C5	4.12	72.8
H6,H6′/C6	3.80, 3.72[a]	64.4[a]

[a] These chemical shifts correspond to the residue that is non-sulfated at the 6-O position.
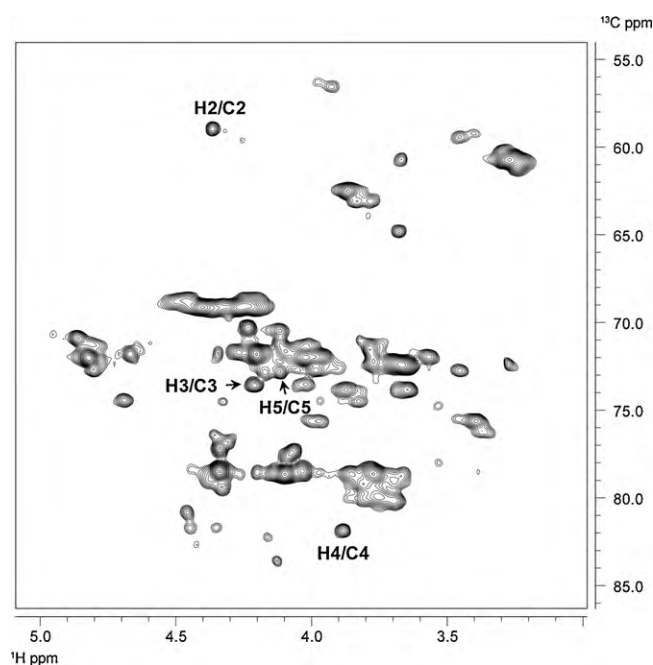

Fig. 5. HSQC spectrum of PI-HSNAc$_{ox}$. NMR assignments for the oxidized residue are indicated close to the relative contours.

results showed that some acetylated species have 16 Da higher mass than the corresponding non-oxidized N-acetylglucosamine species (Supplementary Fig. 5). This observation further substantiates our claim of an oxidized –COOH moiety present at C1 of the reducing end N-acetylglucosamine.

Finally, to confirm whether our observations on the model compound (PI-HSNAc) could be extended to unfractionated heparin, UFH lot 2 was subjected to oxidation with potassium permanganate. In unfractionated heparin samples the amount of N-acetylglucosamine at the reducing end is usually very low (below 1% of the total glucosamine content, as estimated by NMR). Therefore, HSQC experiments of heparin acquired with a sufficient number of scans allow detection of a small peak at 5.20/93.4 ppm belonging to α reducing N-acetylglucosamine (Fig. 6A). Potassium permanganate oxidation of UFH lot 2 caused disappearance of the α reducing N-acetylglucosamine signal and appearance of cross peaks at 4.37/58.9 ppm and 3.88/81.8 ppm (Fig. 6B). This result demonstrates that, similar to the situation for PI-HSNAc, potassium permanganate oxidation of unfractionated heparin results in the formation of an N-acetylglucosaminic acid residue at the reducing end of the chain.

4. Discussion

In a monodimensional proton NMR spectrum, heparin shows a distinct signal at around 2.04 ppm that arises from the methyl (CH$_3$) protons of the N-acetylglucosamine residues. The importance of signals detected in the 2.04–2.20 ppm region was highlighted during the 2008 heparin crisis, when heparin samples contaminated with oversulfated chondroitin sulfate (OSCS) could be identified by a distinct signal at ~2.16 ppm (Guerrini et al., 2008) observed in their ^{1}H NMR spectrum.

Our investigation into the identity of the species or set of species that give rise to the NMR signal at 2.10 ppm was necessitated by the fact that the current USP Monograph for heparin states that, among other criteria, the material should not present any unidentified signals above a specified threshold, between 2.10–3.00 ppm in the ^{1}H NMR spectrum. The NMR analysis of many heparin samples indicated that the 2.10 ppm signal was at least somewhat preva-
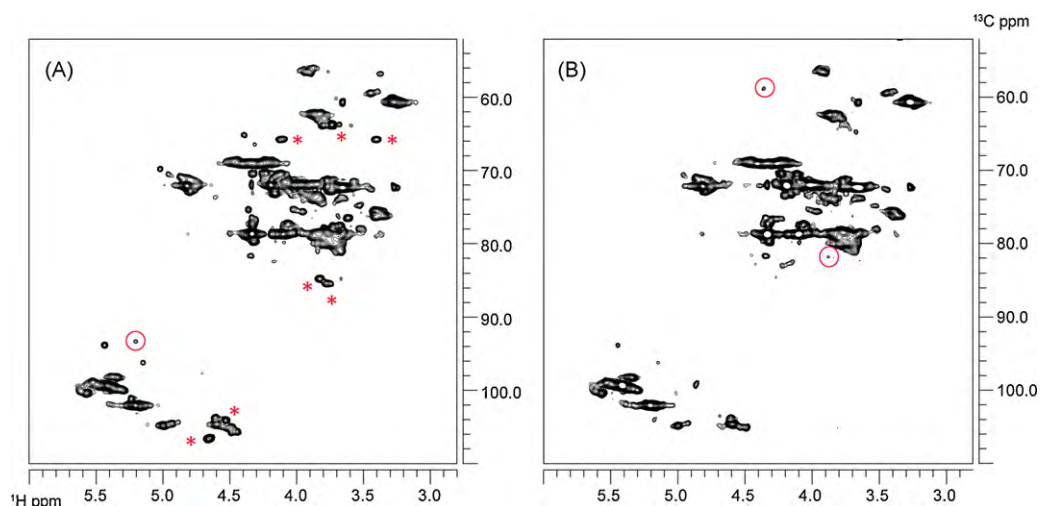
Fig. 6. (A) HSQC spectrum of UFH heparin lot 2. The H1/C1 signal of GlcNAc α reducing end is circled. (B) HSQC spectrum of UFH heparin lot 2 treated with KMnO₄. The H1/C1 signal of GlcNAc α reducing end disappeared, while signals at 4.37/58.9 and 3.88/81.8 ppm appeared (see circled signals). Signals due to the linkage region (indicated with * in A) are also missing.

lent amongst heparin lots, including being present in the European Directorate for the Quality of Medicines heparin reference standard.

It is reasonably well known that modifications can be introduced within the heparin chain based on chemical reactivities of the functional groups present on the chains (Conrad, 1998). A common example is desulfation in the presence of basic/acidic conditions. Furthermore, the application of extraction and purification procedures during the manufacture of heparin has been reported to introduce minor modifications within the heparin chains. For instance, alkali treatment of heparin can induce conversion of 2-*O*-sulfo iduronic acid residue to epoxide, which can be further transformed into non-sulfated iduronic acid or to galacturonic acid residues (Jaseja, Rej, Sauriol & Perlin, 1989). Additionally, alkaline conditions can result in C-2 epimerization of glucosamine–mannosamine residues at the reducing end of chains (Toida et al., 1996), or conversion of the 3-*O*-sulfo glucosamine residues into *N*-sulfo-aziridine derivatives (Casu & Torri, 1999). Indeed, our own experience in the characterization of pharmaceutical grade heparins indicates formation of 1,6-cyclic structures and peeling of monosaccharides at the reducing end as a result of the purification process (data not shown).

Therefore, in our current study, we postulated that the signal at 2.10 ppm arises due to the introduction of minor modifications to the heparin chain, occurring during the manufacture of heparin. We first evaluated the commonly used reagents and chemical processes taking place during the heparin manufacturing process. Based on our research, we hypothesized that the strong oxidation conditions applied during the process may result in modified structures that can be responsible for the appearance of the signal at 2.10 ppm. To test our hypothesis we subjected heparin samples to oxidative conditions with hydrogen peroxide and potassium permanganate, two reagents routinely used in heparin purification. As discussed in the results section above, the ¹H NMR spectrum of the hydrogen peroxide treated samples did not shown the appearance of the signal at 2.10 ppm, but the signal was present in the permanganate treated samples.

Based on this initial observation, we conducted additional experiments to investigate the potential structural modifications resulting from potassium permanganate oxidation of heparin and heparin-like model compounds, which could give rise to the signal at 2.10 ppm. Several lines of evidence pointed to the fact that the major structure which gives rise to the signal at 2.10 ppm arises from modification of the heparin chain, specifically at the *N*-acetylglucosamine moiety. Also, we found that oxidative treatment

of materials rich in internal *N*-acetylglucosamine does not result in the increase of species presenting NMR signals at 2.10 ppm; conversely, treatment of heparin-like model compounds rich in reducing end *N*-acetylglucosamine does result in the production of such species. Accordingly, we observe a dramatic increase in the signal at 2.10 ppm (Fig. 2). Furthermore, concomitant with the increase in signal at 2.10 ppm, there is a corresponding decrease in the signals associated with the α and β forms of the reducing end *N*-acetylglucosamine. To further extend the analysis, multidimensional NMR analysis was completed on defined heparin-like compounds subjected to oxidative conditions with potassium permanganate (KMnO₄). The NMR analysis of one such compound, PI-HSNAc_ox (Table 1), allowed the identification of the major structure which gives rise to the signal at 2.10 ppm. Chemical shift assignments for the oxidized residue (*N*-acetylglucosaminic acid) are provided in Table 2.

Therefore, based on the available evidence, we would propose the following scheme for the formation of such species (Fig. 7). Oxidation agents, such as KMnO₄, react with the reducing *N*-acetyl glucosamine moieties to generate a modified *N*-acetylglucosaminic acid residue (Structure A) at the reducing end of the heparin chain. In this situation, the newly formed signal at 4.37 ppm/58.9 ppm in the HSQC spectrum can be assigned to the proton at the C2 position of the newly generated *N*-acetylglucosaminic acid. It is also possible
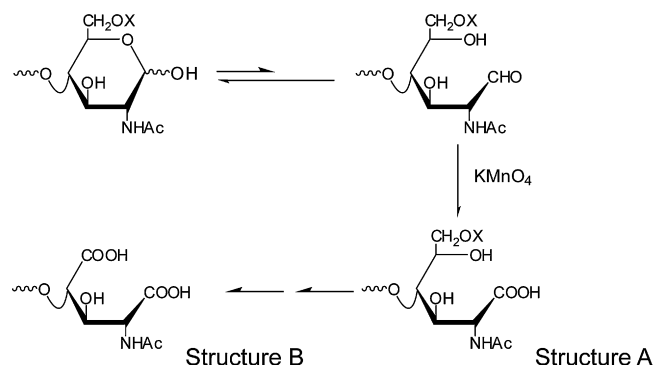

Fig. 7. Reaction scheme outlining the formation of Structure A (*N*-acetylglucosaminic acid; where X = H or SO₃) generated as a result of potassium permanganate oxidation at the reducing end of chains. It is possible that further oxidation of Structure A (if X = H) may result in the formation of a dicarboxylic acid (Structure B). However, Structure B is a proposed structure, and no confirmation of this structure is provided at present.

that further oxidation of this structure may occur, resulting in the formation of a dicarboxylic acid (Structure B), however no confirmation of this structure is provided at present. This scheme (Fig. 7), also explains why the appearance of the signal at 2.10 ppm in the ^{1}H NMR spectrum is dependent on the presence of reducing end *N*-acetylglucosamine. Finally, since the formation of these structures results from oxidation conditions, we anticipate that other oxidation conditions, beyond potassium permanganate, could also potentially result in the formation of such structures.

In conclusion, we find that oxidation conditions result in the conversion of *N*-acetylglucosamine residues at the reducing end of heparin chains to an *N*-acetylglucosaminic acid which yields a characteristic signal at 2.10 ppm in the ^{1}H NMR spectrum of the heparin. Therefore, this signal does not arise from an impurity or contaminant present within heparin, but rather represents a part of the heparin chain itself. Thus, the data presented here should enable the confirmation of the presence of such species in the heparin produced by manufacturers employing oxidative steps.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.05.038.

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