Modification degrees at specific sites on heparan sulphate: an approach to measure chemical modifications on biological molecules with stable isotope labelling

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INTRODUCTION

Chemical modification of biological molecules is a general mechanism for cellular regulation. A quantitative approach has been developed to measure the extent of modification on HS (heparan sulphates). Sulphation on HS by sulphotransferases leads to variable sulphation levels, which allows cells to tune their affinities to various extracellular proteins, including growth factors. With stable isotope labelling and HPLC-coupled MS, modification degrees at various O-sulphation sites could be determined. A bovine kidney HS sample was first saturated in vitro with $^{34}$S by an OST (O-sulphotransferase), then digested with nitrous acid and analysed with HPLC-coupled MS. The $^{34}$S-labelled oligosaccharides were identified based on their unique iso- tope clusters. The modification degrees at the sulphotransferase recognition sites were obtained by calculating the intensities of isotopic peaks in the isotope clusters. The modification degrees at 3-OST-1 and 6-OST-1 sites were examined in detail. This approach can also be used to study other types of chemical modifications on biological molecules.

Key words: chemical modification, heparan sulphate, MS, modification degree, stable isotope, sulphotransferase.

MATERIALS AND METHODS

Materials

Bovine kidney HS, PEP (phosphoenolpyruvate), pyruvate kinase, inorganic pyrophosphatase and sulphurylase were purchased from Sigma (St. Louis, MO, U.S.A.). HS sulphotransferases 3-OST-1 and 6-OST-1 were cloned, expressed and purified with a baculovirus expression system as described previously [21]. The stable

Abbreviations used: aManR, 2,5-anhydromannitol; DBA, dibutylamine; GlcNS, N-sulphated glucosamine; HexA, hexuronic acid; HS, heparan sulphate(s); IdoA, l-iduronic acid; LC, liquid chromatography; 3-OST, 3-O-sulphotransferase, PAPS, 3′-phosphoadenosine 5′-phosphosulphate; TIC, total ion current; XIC, extracted ion current.

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Figure 1  A schematic view of using stable isotope labelling to determine the modification degree at a specific site on a biological molecule

(A) A modification degree (d) at a specific site on a biological molecule. \( \text{ modification introduced in vivo; } \), stable isotope containing modification introduced in vitro. (B) After a sulphation site is saturated with \( ^{34} \text{S} \) in vitro, the \( m/z \) cluster of an oligosaccharide that carries this site can be considered to be a composite of two. One \( m/z \) cluster belongs to the portion of the oligosaccharide generated in vivo (hatched area) and the other belongs to the portion generated in vitro (dark grey area). Intensities of isotopic peaks \( M \) and \( M+2 \) are \( I_1 \) and \( I_3 \) respectively. The enhancement of peak \( M+2 \) after the \( ^{34} \text{S} \) saturation is \( \Delta I_3 \).

isotope \( ^{34} \text{S} \) was obtained from Isonics Corp. (Columbia, MD, U.S.A.). PAPS was prepared as described previously [22]. APS kinase was a gift from Dr I. Segel (University of California, Davis, CA, U.S.A.).

Stable isotope labelling of HS to saturation

The labelling buffer (2×) contained 50 mM Mes (pH 7.0), 1% (w/v) Triton X-100, 5 mM MgCl\(_2\), 5 mM MnCl\(_2\), 2.5 mM CaCl\(_2\), 0.075 mg/ml protamine chloride and 1.5 mg/ml BSA. For a typical 20 \( \mu l \) saturation labelling reaction, 10 \( \mu l \) of HS, 10 \( \mu l \) of labelling buffer, 70 ng of the expressed sulphotransferase, 2 \( \mu l \) of PAPS (3 mM) and an appropriate amount of water were mixed. The mixture was then incubated for 2 h at 37°C. The modified HS was purified on a DEAE column.

Digestion of HS with nitrous acid

Nitrous acid digestion was performed at pH 1.5 to cleave exclusively at GlcNS (N-sulphated glucosamine) residues. Nitrous acid solution was prepared by taking the supernatant of a mixture of equal volumes of 0.2 M Ba(NO\(_2\))\(_2\) and 0.2 M H\(_2\)SO\(_4\). The nitrous acid solution was used within 10 min of preparation. To perform nitrous acid digestion, 10 \( \mu l \) of nitrous acid was added to 10 \( \mu g \) of HS sample and the mixture was held at room temperature (23°C) for 10 min. The reaction was then neutralized with 1 M NaOH and reduced with 1 M NaBH\(_4\) in 0.01 M NaOH at room temperature for 15 min. Residual NaBH\(_4\) was eliminated by the addition of 1 \( \mu l \) of 1 M H\(_2\)SO\(_4\), and the pH was adjusted to approx. 7.0.

Capillary LC (liquid chromatography)/MS

HPLC–MS (HPLC-coupled MS) was described previously [22,23]. Separations were performed on an Ultimate capillary HPLC workstation (Dionex, Sunnyvale, CA, U.S.A.), employing DBA (dibutylamine) as an ion-pairing agent. A gradient elution was performed, using a binary solvent system composed of water (eluent A) and 70% methanol (eluent B), both containing 8 mM acetic acid and 5 mM ion-pairing reagent. HPLC separations were performed on a 0.3 mm × 250 mm C\(_{18}\) column (MS; 5 \( \mu m \)) at a rate of 5 \( \mu l/min \) and a sample volume of 6.3 \( \mu l \). The elution profile was 0% B for 5 min, 6% B for 19 min, 18% B for 17 min, 34% B for 13 min and 55% B for 16 min. After each run, the column was washed with 90% B for 15 min and equilibrated with 100% A for 28 min.

Mass spectra were acquired on a Mariner BioSpectrometry Workstation ESI (electrospray ionization) time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA, U.S.A.). Nitrogen was used as a desolvation gas as well as a nebulizer. Conditions for electrospray ionization MS were as follows: nebulizer gas (N\(_2\)) flow rate, 1 litre/min; nozzle temperature, 140°C; drying gas (N\(_2\)) flow rate, 0.6 litre/min; spray tip potential, 2.8 kV; and nozzle potential, 70 V. Negative ion spectra were acquired every 4 s by scanning \( m/z \) from 40 to 4000. Total ion chromatograms and mass spectra were processed with the Data Explorer software version 3.0.

Computer simulation of the natural isotope clusters of HS oligosaccharides

The natural isotope clusters of oligosaccharides were simulated with Isotope Calculator in the Data Explorer software. After the isotope cluster of a oligosaccharide was generated, the intensities of all isotopic peaks were normalized to that of the monoisotopic peak.

RESULTS

Theoretical calculation of a modification degree at a site of interest on a biological molecule

If a chemical modification on a biological molecule is not complete \( \text{in vivo} \), it is possible to complete the modification \( \text{in vitro} \)
with a stable isotope-containing a donor, which causes the enhancement of a specific peak in the isotope cluster of the molecule. For example, if a stable isotope such as $^{18}$O or $^{34}$S is used for labelling, the $m/z$ cluster of the molecule can be considered as a composite of the two. On labelling of an HS oligosaccharide with $^{34}$S by a sulphotransferase, the monoisotopic peak $M$ in the cluster is not affected by the process and thus represents the in vivo-modified portion; in contrast, the enhancement of isotopic peak $M + 2$ represents the in vitro-modified portion (Figure 1B). Assumming that the intensity of the monoisotopic peak $M$ is $I_1$ and the enhancement of peak $M + 2$ is $\Delta I_1$, the modification degree ($d$) at the sulphotransferase recognition site will be

$$d = \left[ I_1/(I_1 + \Delta I_1) \right] \times 100\% \quad (1)$$

Because the natural abundance of each stable isotope is relatively constant, the initial intensity of the peak $M + 2$ is a constant too. The relative initial $I_1$ value of the oligosaccharides could be obtained through computer simulation on the basis of natural abundance of each stable isotope.

$m/z$ values of nitrous acid-generated HS oligosaccharides

At pH 1.5, nitrous acid selectively attacks GlcNS on a HS chain, whereas N-acetylated and unmodified glucosamine residues are not affected [24]. Nitrous acid treatment converts a GlcNS residue into 2,5-anhydromannose and results in the cleavage of the corresponding glucosaminidic linkage. Further reduction by NaBH$_4$ converts the unstable 2,5-anhydromannose into aMan$_n$ (2,5-anhydromannitol). The net result is various oligosaccharides with HexA (hexuronic acid) at the non-reducing ends and aMan$_n$ at the reducing ends (Figure 2); therefore a nitrous acid-generated oligosaccharide can be considered as a collection of one HexA-aMan$_n$, a number of internal HexA-GlcNAc, a number of internal HexA-GlcNH$_2$ and a number of sulphates. Based on the major stable isotopes of $^{12}$C, $^1$H, $^{18}$O, $^{14}$N and $^{32}$S, HexA-aMan$_n$ ($C_{12}H_{20}O_{11}$), internal HexA-GlcNAc ($C_{14}H_{21}O_{10}N_1$) and internal HexA-GlcNH$_2$ ($C_{14}H_{20}O_{10}N_2$) have molecular masses of 340.09, 379.11 and 337.10 Da respectively and sulphation increases the mass by 79.96 (addition of S$_3$O$_3$). For convention, a nitrous acid-generated oligosaccharide can be designated as

$$d p X - (p-n)Ac - q S$$

where $X$ is the number of sugar residues, $p$ is the number of internal disaccharides, $X = 2(p + 1)$, $n$ is the number of free amino groups, $q$ is the number of sulphates, Ac stands for the acetyl group and S stands for sulphate. Accordingly, the $m/z$ value of a negatively charged oligosaccharide will be

$$m/z = [340.09 + 379.11(p - n) + 337.10n + 79.96q - z]/z \quad (2)$$

The calculated value should correspond to the monoisotopic peak $M$ in the $m/z$ cluster of an oligosaccharide (Figure 1B, and Supplemental Files 1 and 2 at http://www.BiochemJ.org/bj/389/bj3890383add.htm).

The modification degrees at the 3-OST-1 sites on bovine kidney HS

Bovine kidney HS was first saturated with a stable isotope $^{34}$S by 3-OST-1 (see Supplemental Files 5 and 7 at http://www.BiochemJ.org/bj/389/bj3890383add.htm) and then digested with nitrous acid at pH 1.5. The digest was analysed with HPLC–MS. Two $^{34}$S-labelled oligosaccharides were found with greatly enhanced isotopic peaks $M + 2$ at $m/z$ 439.59 and 479.57. The monoisotopic peak $M$ of the two isotope clusters was at $m/z$ 438.59 and 478.59 respectively. The distance between neighbouring peaks in the two clusters was 0.5 unit, indicating the double-charged ions ($z = 2$). After these values were fitted into eqn (2), the parameters were found to be $X = 4$, $p = 1$, $n = 0$ and $q = 2$ or 3. The two oligosaccharides were then designated as dp4-1Ac-2S and dp4-1Ac-3S (Table 1).

An XIC (extracted ion current) chromatogram was then obtained at $m/z$ 439.59 and 479.57 from the TIC (total ion current) chromatogram (Figure 3A). Based on peak areas in XIC, the two
Table 1 Identification of 3-OST-1- and 6-OST-1-labelled oligosaccharides on bovine kidney HS

The relationship between the m/z values of the monoisotopic peaks, the number of internal disaccharides (p), the number of NH₂-containing disaccharides (n) and the number of sulphates (q) was calculated based on eqn (2), m/z = \([340.09 + 379.11(p - n) + 337.10n + 79.96q - z]/z\).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Monoisotopic peak (m/z)</th>
<th>Elution time (min)</th>
<th>z</th>
<th>n</th>
<th>p</th>
<th>q</th>
<th>Formula</th>
</tr>
</thead>
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<td>0</td>
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<td>2</td>
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<tr>
<td></td>
<td>478.59</td>
<td>60.3</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>dp4-1Ac-3S</td>
</tr>
<tr>
<td>6-OST-1</td>
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<td>41.7</td>
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<td>0</td>
<td>0</td>
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<td>dp2-2S(a)*</td>
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<td></td>
<td>499.05</td>
<td>44.2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>dp2-2S(b)*</td>
</tr>
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<td>708.19</td>
<td>55.0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>dp2-3S:1DBA*</td>
</tr>
</tbody>
</table>

* Only major molecular species were listed.

Oligosaccharides were estimated to be in the ratio 5:1 (semi-quantitative estimation, because the peak intensity in an XIC is not only related to the quantity but also to the charge state and ionization condition). Sodium adducts were observed for both oligosaccharides and a sulphate loss was observed for the second oligosaccharide (Figure 3B, and Supplemental File 3 at http://www.BiochemJ.org/bj/389/bj3890383add.htm). The intensities of the isotopic peaks in the m/z clusters were directly obtained from data acquisition. The relative intensities of isotopic peak \(M + 2(I_s)\) of the two labelled oligosaccharides were found to be 6.12 and 27.86 respectively (Table 2).

The isotope clusters of dp4-1Ac-2S (C₂₆H₄₁O₂₈N₁S₂) and dp4-1Ac-3S (C₂₆H₄₁O₃₁N₁S₃) were also generated with the Isotope Calculator according to the natural abundance of each stable isotope (Figure 3C), and the initial relative intensities of the peaks \(M + 2\) were determined to be 0.20 and 0.25 respectively. The modification degrees at these two 3-OST-1 sites were then calculated to be 14 and 40% respectively (Table 2) using eqn (1).

Figure 3 The modification degrees at 3-OST-1 sites

(A) TIC of the nitrous acid-digested, ³⁴S³/³⁵S³-OST-1-saturated HS sample is followed by XIC at m/z 439.59 ± 0.2 and 479.59 ± 0.2. The XIC had peaks at T53.3 and T60.3. (B) The mass spectra and m/z clusters of the two 3-OST-1-labelled oligosaccharides. The two oligosaccharides were identified to be a dp4-1Ac-2S and a dp4-1Ac-3S. The peak at T60.3 in the XIC exhibited m/z 439.60, 479.57 and 490.58. The m/z 439.60 was caused by the loss of a sulphate from m/z 479.57. (C) The isotope clusters of dp4-1Ac-2S and dp4-1Ac-3S, generated by a computer program according to the natural abundances of stable isotopes.

Modification degrees at 6-OST-1 sites on bovine kidney HS

The modification degrees at 6-OST-1 sites on bovine kidney HS were also determined (see Supplemental Files 6 and 8 at http://www.BiochemJ.org/bj/389/bj3890383add.htm). Three oligosaccharides were labelled by 6-OST-1 (Figure 4). The three M + 2 peaks in the isotope clusters of the oligosaccharides were at m/z 501.05, 501.05 and 710.19. An XIC (Figure 4A) showed the proportion of the three oligosaccharides to be 20:1.5:1 according to the peak intensities (semi-quantitative estimation).

In the mass spectra, sodium and DBA adducts were observed for the three oligosaccharides (Figure 4B). The distance between the

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neighbouring peaks in all isotope clusters was 1 unit, indicating single-charged ions ($z=1$). The first oligosaccharide had two isotope clusters with the monoisotopic peak $M$ at 499.05 and 628.22 respectively. Since the difference between the two values was 129.17, the second $m/z$ cluster was probably from the complex between this oligosaccharide and the ion-pairing reagent DBA [NH(C$_{4}$H$_{9}$)$_{2}$; molecular mass, 129.15 Da] (see Supplemental File 4 at http://www.BiochemJ.org/bj/389/bj3890383add.htm). By fitting the data into eqn (2), the parameters for this oligosaccharide were found to be $X=2$, $p=0$, $n=0$ and $q=2$; therefore the oligosaccharide was a dp2-2S (Table 1). The second oligosaccharide had a very similar mass spectrum and was also a dp2-2S. The first oligosaccharide was then designated dp2-2S(a) and the second one was designated dp2-2S(b). The third oligosaccharide had two isotope clusters with the monoisotopic peaks $M$ at 708.19 (579.04 + 129.15) and 837.33 [579.03 + (129.15 × 2)], indicating the formation of complexes between the oligosaccharide and one or two molecules of DBA. This oligosaccharide was identified as a dp2-3S (Table 1). The relative intensities of the isotopic peaks $M+2$ of the dp2-2S(a), dp2-2S(b) and dp2-3S:1DBA (complex between the dp2-3S and one DBA) were determined to be 0.80, 0.71 and 1.17 respectively according to their $m/z$ clusters (Table 2).

When the isotope clusters of dp2-2S and dp2-3S:1DBA were simulated with Isotope Calculator (Figure 4C), the initial intensities of the peaks $M+2$ were found to be 0.13 and 0.21 respectively (Table 2). The modification degrees at the three 6-OST-1 sites were then determined to be 60, 63 and 51% respectively (Table 2).

**Table 2  Calculation of the modification degrees (d) at 3-OST-1 and 6-OST-1 sites**

The intensities of isotopic peaks for 34S-saturated oligosaccharides by 3-OST-1 and 6-OST-1 were obtained through data acquisition. The initial intensities of the isotopic peaks were obtained by computer simulation. Peak intensities of monoisotopic peaks were arbitrarily set at 1 unit.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Oligosaccharide formula</th>
<th>Isotopic peak $m/z$</th>
<th>Intensity</th>
<th>Initial intensity</th>
<th>$\Delta d$</th>
<th>$d$ (%)</th>
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</thead>
<tbody>
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<td>dp4-1Ac-2S</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(C$<em>{26}$H$</em>{41}$O$<em>{28}$N$</em>{1}$S$_{2}$) M</td>
<td>439.59</td>
<td>6.12</td>
<td>0.20</td>
<td>5.92</td>
<td>14</td>
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<tr>
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<tr>
<td></td>
<td>(C$<em>{26}$H$</em>{41}$O$<em>{31}$N$</em>{1}$S$_{3}$) M</td>
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<td>27.86</td>
<td>0.25</td>
<td>27.61</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(C$<em>{12}$H$</em>{20}$O$<em>{17}$S$</em>{2}$) M</td>
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<td>0.80</td>
<td>0.13</td>
<td>0.67</td>
<td>60</td>
</tr>
<tr>
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<td>dp2-2S (b)</td>
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<td>63</td>
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<td>(C$<em>{12}$H$</em>{20}$O$<em>{17}$S$</em>{2}$) M</td>
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<td>1.17</td>
<td>0.21</td>
<td>0.96</td>
<td>51</td>
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</table>

![Figure 4](http://www.BiochemJ.org/bj/389/bj3890383add.htm)
DISCUSSION

There were two different sulphation sites for 3-OST-1 on bovine kidney HS. The modification degree at the site in dp4-1Ac-2S was 14%, whereas the modification degree at the site in dp4-1Ac-3S was only 4%. Considering that the amount of dp4-1Ac-2S was approx. 5-fold that of dp4-1Ac-3S (Figure 3A), it is clear that most of the oligosaccharides modified by 3-OST-1 in vivo were dp4-1Ac-2S. Recently, the 3-OST-1 knockout mice were reported to have normal anticoagulant activities [25], suggesting that 3-OST-1-modified oligosaccharides might have other biological functions. The fact that the modification degrees at 3-OST-1 sites were significantly increased in retinoic acid-stimulated F9 embryonal carcinoma cells, indicated that 3-OST-1 might have a role in the differentiation of this cell line [26]. Overall, the modification degrees at the two 3-OST-1 sites on bovine kidney HS were very low, which leaves a relatively larger portion of the sites in the unmodified state and may allow a rapid increase in the 3-O-sulphation levels in response to stimuli such as retinoic acid [26].

On the other hand, 6-OST-1 had three different sulphation sites on bovine kidney HS. All modification degrees at these sites were more than 50%, which are significantly higher than those at the 3-OST-1 sites. The higher modification degrees at 6-OST-1 sites left less room for further addition of sulphates. However, it was found that the modification degrees at the 6-O sites can also be adjusted by HS 6-O-endosulfatase [17] that takes away sulphates from these 6-O sites [18, 20]. This regulation of sulphation levels by sulphotransferases/sulphatases is very similar to the regulation of phosphorylation levels by kinases/phosphatases [2].

We found that stable isotope labelling and LC/MS have been applied recently for measuring the phosphorylation levels on proteins and peptides [27,28]. The novelty of our method lies in saturating the unmodified site with a stable isotope in vitro, which renders the site modified in vitro chemically identical with the site modified in vivo. Subsequently, both sites will appear in a same isotope cluster during LC/MS, and the ratio between the in vivo- and in vitro-modified sites can be quantitatively determined.

In summary, sulphation degrees at specific sites on HS are important parameters for cells to tune their affinities for various extracellular proteins; the measurement of sulphation degrees opens a new avenue to study the HS structure–function relationship; stable isotope labelling and LC/MS can also be used to measure other chemical modifications on biological molecules.

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