



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Photochemical depolymerisation of dermatan sulfate and analysis of the generated oligosaccharides

Citation for published version:

Panagos, CG, August, DP, Jesson, C & Uhrin, D 2016, 'Photochemical depolymerisation of dermatan sulfate and analysis of the generated oligosaccharides' *Carbohydrate polymers*, vol. 140, pp. 13-19. DOI: 10.1016/j.carbpol.2015.11.078

Digital Object Identifier (DOI):

[10.1016/j.carbpol.2015.11.078](https://doi.org/10.1016/j.carbpol.2015.11.078)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Carbohydrate polymers

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



1 Photochemical depolymerisation of dermatan sulfate and analysis of the generated
2 oligosaccharides.

3 Charalampos G. Panagos,^{a,b} David P. August,^a Christine Jesson,^b Dušan Uhrín^{a,*}

4 ^a EastChem School of Chemistry, The University of Edinburgh, King's Buildings, David Brewster Road ,
5 Edinburgh EH9 3FJ, United Kingdom.

6 ^b GlycoMar Ltd, European Centre for Marine Biotechnology, Oban, Scotland, PA37 1QA, United Kingdom.
7

8 *To whom correspondence should be addressed: EaStCHEM School of Chemistry, Joseph Black Bldg.,
9 The King's Buildings, University of Edinburgh, Edinburgh, EH9 3FJ, United Kingdom. Tel.: 44-131-650-
10 4742; Fax: 44-131-650-7155; E-mail: dusan.uhrin@ed.ac.uk.
11

12 **Abstract**

13 Radical depolymerisation is the method of choice for the depolymerisation of glycosaminoglycans
14 (GAGs), especially when enzymatic depolymerisation cannot be performed due to the lack of suitable
15 enzymes. The established Fenton type free radical depolymerisation generates radicals from a solution
16 of H₂O₂ in the presence of Cu²⁺ or Fe²⁺. When applied to dermatan sulfate (DS), the Fenton type
17 depolymerisation of DS (Panagos, Thomson, Bavington & Uhrin, 2012) produced exclusively
18 oligosaccharides with reducing end GalNAc, which was partially oxidized to acetylgalactosaminic acid.
19 We report here the results of the TiO₂ catalyzed photochemical depolymerisation of DS. NMR analysis of
20 these DS oligosaccharides revealed the presence of reducing end IdoA, observed for the first time. The
21 reducing end acetylgalactosaminic acid was also detected. The photochemical depolymerisation method
22 thus enables preparation of new types of GAG oligosaccharides suitable for further biochemical and
23 biological investigation.
24

25 **Highlights**

- 26 • Free radical photochemical depolymerisation generates GAG oligosaccharides.
27 • The structures of DS oligosaccharides were studied by NMR.
28 • Oligosaccharides with uronic acid as the reducing end monosaccharides were found.
29 • Acetylgalactosaminic reducing end monosaccharides were also generated.
30 • No desulfation of the generated oligosaccharides was found.
31

32 **Keywords**

33 NMR; glycosaminoglycans; dermatan sulfate; photochemical depolymerisation.
34
35

36 **Chemical compounds studied in this article**

37 Phosphate-buffered saline (PubChem CID: 24978514); Titanium dioxide (PubChem CID: 26042),
38 Ethylenediaminetetraacetic acid (PubChem CID: 6049), Trimethylsilyl propionate (PubChem CID:
39 519321), Dermatan sulfate (Pubchem CID:32756).

40 **Abbreviations**

41 GAGs, glycosaminoglycans; LMWH, low molecular weight heparin; DS, dermatan sulfate; GalNAc, N-
42 acetylgalactosamine; IdoA, iduronic Acid; HSQC, Heteronuclear single quantum coherence; TOCSY, Total
43 correlation spectroscopy; HMBC, Heteronuclear multiple bond coherence;

44

45 **1. Introduction**

46

47 Glycosaminoglycans (GAGs) are a biologically important class of carbohydrates usually found on cell
48 surfaces in the form of proteoglycans (Dietrich, Sampaio, Montesdeoca & Nader, 1980; Mathews, 1975;
49 Medeiros, Mendes, Castro, Baú, Nader & Dietrich, 2000). They are essential for a variety of cell-cell
50 signaling events, such as cell growth and cell adhesion (Singer, 1992). As long unbranched
51 polysaccharides, GAGs also participate in the construction of connective tissues (Comper & Laurent,
52 1978; Mathews, 1975), such as cartilage. The ability of GAGs to be involved in such a variety of
53 biochemical processes is underpinned by their inherent structural heterogeneity.

54 GAGs are built from repeating disaccharide units, which typically consist of an uronic acid (iduronic
55 acid or glucuronic acid) and a hexosamine (galactosamine or glucosamine). Furthermore, these
56 disaccharide units can be sulfated at different sites and the position and type of the glycosidic linkage
57 also varies between GAG types. These attributes lead to structural variety within the GAG family.
58 Although not random, the epimerization and sulfation events are not regular, creating large
59 heterogeneity within a single GAG type. For example, during the processing of heparan, the precursor of
60 heparan sulfate (HS), various enzymes including N-deacetylases/N-sulfotransferases, as well as 6-O- and
61 3-O sulfotransferases and C-5 epimerases, modify its structure, though these modifications are neither
62 random or complete (Vreys & David, 2007). The resulting macroscopic organization of HS shows a typical
63 'block structure' containing extensive structural heterogeneity (Gallagher, 2006).

64 In order to study the biology and pharmacology of these heterogeneous polysaccharides it is
65 necessary to identify specific structures within their chains, which are responsible for their distinct
66 biological properties (Toida, Sato, Sakamoto, Sakai, Hosoyama & Linhardt, 2009). For example, the study
67 of heparin oligosaccharides revealed the existence of a specific pentasaccharide sequence responsible
68 for the binding of heparin to antithrombin (Munoz & Linhardt, 2004). GAG oligosaccharides are also
69 important in their own right, e.g. low molecular weight heparin (LMWH) (Choay, Lormeau, Petitou, Sinaÿ
70 & Fareed, 1981), has almost completely replaced unfractionated heparin as a pharmaceutical (Hileman,
71 Smith, Toida & Linhardt, 1997). Low molecular weight dermatan sulfates (DS) also show increased
72 bioavailability without the loss of bioactivity (Dol et al., 1990; Legnani et al., 1994).

73 Smaller GAG oligosaccharides can lead to a reduction in the adverse effects sometimes associated
74 with full-length polysaccharides. For example, LMWH decreases the occurrence of heparin induced
75 thrombocytopenia due to less efficient interaction with platelet factor 4 (Gray, Mulloy & Barrowcliffel,
76 2008). LMWH also reduces the risk of osteoporosis, another side effect of heparin (Matzsch, Bergqvist,
77 Hedner, Nilsson & Ostergaard, 1990; Monreal, Vinas, Monreal, Lavin, Lafoz & Angles, 1990). Another
78 example is the depolymerisation of fucosylated chondroitin sulfate, fCS, from sea cucumber (Panagos et
79 al., 2014; Suzuki, Kitazato, Takamatsu & Saito, 1991; Wu, Xua, Zhao, Kang & Ding, 2010; Wu et al., 2013;
80 Yang, Wang, Jiang, Lv, Zhang & Lv, 2015), which is critical for antithrombotic applications, due to an
81 undesired platelet aggregation associated with long polysaccharide chains (Wu, Xu, Zhao, Kang & Ding,
82 2010b).

83 Depolymerisation of GAGs and the production of fully characterised oligosaccharides is therefore
84 an essential process in evaluation of the biological potential of these molecules. A variety of different
85 depolymerisation methods, characterized by specific advantages and limitations, have therefore been
86 developed to date. The most widely used is enzymatic depolymerisation, although this method also has
87 its limitations. For example, highly sulfated GAGs, which are typical for marine species, are resistant to
88 enzymatic depolymerisation (Vieira, Mulloy & Mourao, 1991). Moreover, oligosaccharides generated by
89 heparanases and chondroitinases contain an unsaturated uronate residue as the non-reducing end
90 monosaccharide and a hexosamine as the reducing end monosaccharide (Pervin, Gallo, Jandik, Han &
91 Linhardt, 1995). Finally, depolymerisation enzymes can be very expensive, e.g. one mg of chondroitinase
92 ABC lyase, which can deliver between 50 to 250 units, can cost much more than £ 1000.

93 Other depolymerisation methods include acid hydrolysis, solvolysis and ultrasonication. Acid
94 hydrolysis is a chemically based approach which involves both the depolymerisation and the desulfation
95 of the polysaccharide (Mourao et al., 1996) and for that reason it is not widely used on sulfated GAGs.
96 Solvolytic depolymerisation has also been shown to be effective on GAGs but it also involves
97 desulfonation of the polysaccharide chains and is also known to structurally modify the oligosaccharide
98 products (Toida, Sato, Sakamoto, Sakai, Hosoyama & Linhardt, 2009). Ultrasonic depolymerisation is
99 unique among the depolymerisation methods, as it is a mechanical method, which has been employed
100 for the depolymerisation of hyaluronic acid (Miyazaki, Yomota & Okada, 2001). Although this technique
101 generates lower molecular weight GAGs with unmodified chains, its application is limited because of the
102 size of produced species ($MW > 10^5$ Da), which are much bigger than those generated by other methods.

103 One of the more widely used chemical depolymerisation methods suitable for GAGs is the free
104 radical Fenton-type depolymerisation. It has been used not only to generate oligosaccharides from
105 heparin (Nagasawa, Uchiyama, Sato & Hatano, 1992), chondroitin and dermatan sulfate (Ofman, Slim,
106 Watt & Yorke, 1997), and hyaluronate (Uchiyama, Dobashi, Ohkouchi & Nagasawa, 1990), but also
107 marine GAGs (Wu, Xu, Zhao, Kang & Ding, 2010a). However, Fenton-type depolymerisation reaction is
108 known to preferentially degrade unsulfated IdoA in heparin (Nagasawa, Uchiyama, Sato & Hatano, 1992)
109 and DS (Ofman, Slim, Watt & Yorke, 1997; Panagos, Thomson, Bavington & Uhrin, 2012), thus creating
110 oligosaccharides that have hexosamine at the reducing end, similar to enzymatic depolymerisation. In
111 addition, this reducing end hexosamine can sometimes be oxidized, as seen in heparin and DS, forming
112 acetylglucosaminic acid and acetylgalactosaminic acid, respectively (Panagos, Thomson, Bavington &
113 Uhrin, 2012; Vismara, Pierini, Guglieri, Liverani, Mascellani & Torri, 2007; Vismara et al., 2010).

114 In this work we present a structural analysis of oligosaccharides obtained by a photochemical
115 depolymerisation of a model GAG, DS, and compare them with DS oligosaccharides obtained previously
116 by the Fenton-type depolymerisation (Panagos, Thomson, Bavington & Uhrin, 2012), as both methods
117 use free radicals to cleave the glycosidic bonds. The photochemical depolymerisation has been used
118 recently to depolymerise alginate (Burana-osot, Hosoyama, Nagamoto, Suzuki, Linhardt & Toida, 2009),
119 pectin (Burana-osot, Soonthornchareonnon, Hosoyama, Linhardt & Toida, 2010), K5 heparosan (Higashi
120 et al., 2011) and heparin (Higashi et al., 2012). While the Fenton type depolymerisation generates free
121 radicals from hydrogen peroxide using an iron or copper catalyst, photochemical depolymerisation uses
122 UV light and titanium dioxide to generate radicals in an aqueous environment, making it a very
123 inexpensive technique.

124

125

126

127 **2. Materials and methods**

128

129 2.1 Materials

130

131 Titanium(IV) oxide (TiO₂) anatase powder was purchased from Acros Organics. Porcine DS,
132 containing more than 95% of the repeating disaccharide [-β-D-GalNAc4S-(1 → 4)-α-L-IdoA-(1 → 3)]_n was
133 obtained from Celsus Laboratories. Ethylenediaminetetraacetic acid (EDTA) and trimethylsilyl
134 propionate (TSP) were purchased from Goss Scientific Instruments Ltd. and Aldrich, respectively.

135

136 2.2 Depolymerisation of polysaccharides by photochemical reaction

137 Samples (300 mg) were dissolved in 30 ml of deionised water in a shallow open crystallising dish to
138 a final concentration of 10 mg/ml. 30 mg (1:10) of titanium(IV) oxide (TiO₂) anatase powder (Acros
139 Organics) was added. A magnetic stirrer was added to the sample to ensure the dissolution of
140 atmospheric oxygen in the solution and to prevent settling of TiO₂. The receptacle was placed under a
141 UV light source (125 W low pressure mercury lamp from Photochemical Reactors Ltd). In order to
142 maximise UV absorbance by the TiO₂ the lamp was placed 10 cm from the receptacle at approximately a
143 70° angle. The reaction was carried out at room temperature and was stopped after 34 h, when the
144 reaction progress was deemed to be satisfactory. 100 µl samples were taken at regular intervals (8-10
145 hours) and the progress of the reaction was monitored by HPLC, following the method described below.
146 The samples were centrifuged at 16,100 × g for 5 minutes at room temperature. The supernatant was
147 passed through a 0.22 µm membrane filter for complete removal of the TiO₂ particles. The samples were
148 separated by size exclusion chromatography (SEC) at room temperature for 45 minutes at a flow rate of
149 0.5 ml/min. A Superdex Peptide 10/300 GL size exclusion column attached to a Waters 600 LCD HPLC
150 equipped with a 486 Tunable Absorbance Detector was deployed. PBS (Phosphate Buffered Saline
151 tablets, Fisher Scientific) was used as mobile phase and the UV absorbance was recorded at 218 nm, as
152 the absence of a double bond generated from enzymatic depolymerisation, prevents the use of the
153 absorbance at 232nm. The total reaction mixture after the end of the reaction was centrifuged for 30
154 min at 2,742 × g and the supernatant was passed through a 0.22 µm membrane and freeze-dried.

155 2.3 Fractionation and isolation of the oligosaccharides

156 The freeze-dried material obtained from the depolymerisation reaction was redissolved in 2.5 ml
157 deionised water and fractionated by SECon a BioCAD 700E Workstation FPLC system equipped with a
158 Waters Fraction Collector. Two XK26/100 columns preceded by a XK26/20 guard column (GE healthcare)
159 were packed with Bio-Gel P-10 Fine resin (Bio-Rad Laboratories) and connected in series. Enzymatically
160 cleaved DS tetrasaccharides and hexasaccharides were used as standards. The sample was loaded on a 5
161 ml loop and was run at a flow rate of 0.4 ml/min for a total of 62.5 h, with PBS as the mobile phase. The
162 UV absorbance was recorded at 218 nm and the fractions containing oligosaccharides were collected,
163 pooled and freeze dried.

164 Prior to NMR experiments, the lyophilized samples were desalted using the BioCad 700E system
165 equipped with a XK16/40 column packed with Sephadex G25 superfine (Sigma-Aldrich). Each run was
166 carried out with an injection volume of 5 ml and dH₂O as mobile phase. For each run, the injection loop
167 was first flushed for 6 minutes at 2.5 ml/min and then the flow rate was increased to 4 ml/min for 21
168 minutes and 3 ml fractions were collected. The UV absorbance was recorded at 218 nm. Four to five
169 fractions were pooled together, yielding desalted oligosaccharides in 12-15 ml of solution. The samples
170 were subsequently freeze-dried.

171 2.4 Structural analysis of the fractions by NMR

172 After lyophilisation the samples were dissolved in 99.9% D₂O, (Aldrich, 540 µl) containing deuterated
173 NaH₂PO₄ + HNa₂HPO₄ buffer (10 mM, pH 7.2). A stock solution (20 µl) of EDTA and TSP was added. The
174 stock solution was prepared by dissolving EDTA (4 mg) and TSP (9 mg) in the phosphate buffer (200 µl).
175 The pH was adjusted to 7.2 by adding few drops of a concentrated solution of NaOH in D₂O. All spectra
176 were acquired at 50 °C on an 800 MHz Avance I Bruker NMR spectrometer equipped with a z-gradient
177 triple-resonance TCI cryoprobe. The spectra were referenced (0 ppm) using the ¹H and ¹³C signals of TSP.

178 1D ¹H NMR spectra were acquired using relaxation and acquisition times of 1.5 and 0.4999 s,
179 respectively; 32 scans per spectrum were accumulated. 1D ¹³C NMR spectra with ¹H GARP decoupling
180 were acquired using relaxation and acquisition times of 4 and 0.6816 s, respectively; 31504 scans per
181 spectrum were accumulated in 41 h. The FIDs were zero filled once and a 2 Hz exponential line
182 broadening was applied prior to Fourier transformation. 2D ¹H, ¹³C HSQC spectra were acquired using a
183 modified HSQC sequence. This pulse sequence employs DEPT editing sequence, which ensured that the
184 CH₂ signals appear as negative peaks. This was invaluable for the identification of C6 atoms of GAGs. The
185 polarization transfer was optimised for ¹J_{CH} = 150 Hz for all the experiments and ¹³C adiabatic decoupling
186 was used to decouple the carbon-proton couplings. Spectra were acquired using *t*₁ and *t*₂ acquisition
187 times of 0.011 and 0.1069 s, respectively; 4 scans were acquired into each of 512 *F*₁ complex data points
188 resulting in the total experimental times of 1.5 h per sample.

189 2.5 Fractionation of fucosylated chondroitin sulfate.

190 The samples of fCS was depolymerized and purified as described previously (Panagos et al., 2014).

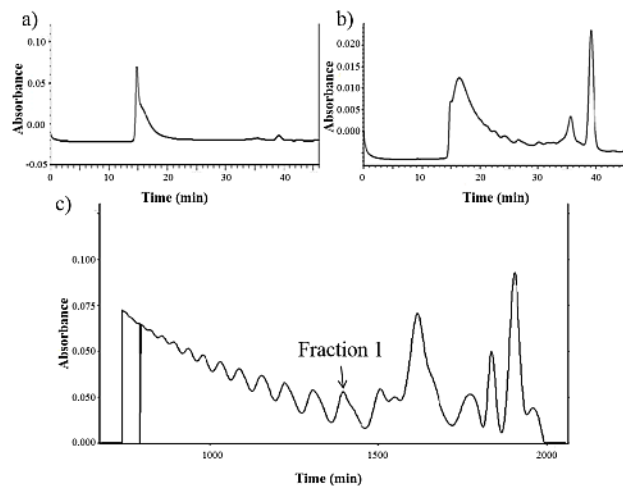
191

192 **3. Results**

193

194 3.1 Fractionation of DS and isolation of the DS oligosaccharides

195 Samples taken from the reaction mixture containing DS and TiO₂ in water as described in detail in 2.2,
196 were analysed periodically to monitor the progress of the reaction. Monitoring by HPLC showed that the
197 photochemical depolymerisation of DS does not progress at a constant rate under the conditions used.
198 As indicated by the chromatogram of Figure 1a, the peak eluting with the column void volume,
199 containing oligosaccharides with M_w>7000 Da is almost intact after the first 24 h. The reaction seems to
200 progressively accelerate and after 34 h the peak eluting with the void volume has decreased
201 significantly. At the same time the presence of smaller oligosaccharides is clearly visible (Figure 1b). The
202 reaction was therefore stopped at this point and the reaction mixture was separated using a SEC
203 column. The SEC chromatogram (Figure 1c) shows separation of fragments; the fraction labelled I,
204 composed from fractions 77-83 (total volume 70 ml) was chosen for structural analysis, since its elution
205 time was similar to that of a DS tetrasaccharide standard, run previously during the column calibration
206 (data not shown). A shoulder visible on the right hand side of peak I is most likely due to co-elution
207 trisaccharide species as discussed later, while the peak eluting at approximately 1500 minutes belongs
208 to the disaccharides fractions. The peaks eluting later contained fragments that when analysed by NMR,
209 were identified as smaller than the disaccharide, potentially monosaccharides or even smaller entities.



210

211 **Figure 1: Chromatograms of the depolymerized DS (a) and (b) show HPLC traces after 24 and 34 hours, respectively. (c)**
212 **Screenshot of the final 1500 minutes of the size exclusion chromatogram of the final reaction mixture obtained after 34 h of**
213 **depolymerisation. The UV absorbance was monitored at 218 nm and the fraction brought forward for the analysis is**
214 **indicated.**

215

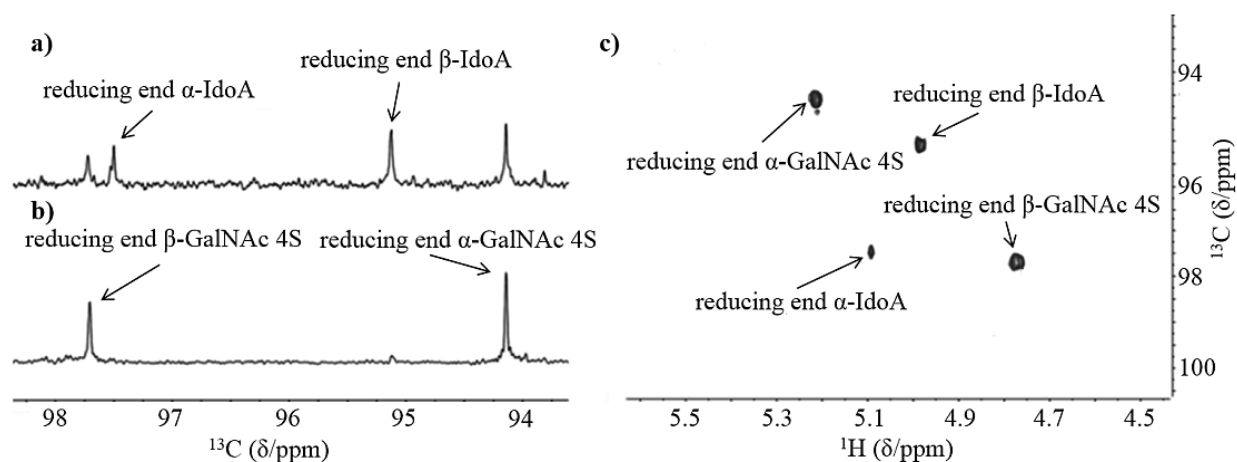
216

217 3.2 NMR analysis of fraction I

218 Fraction I was analyzed by 1D and 2D NMR methods and the structures of the constituting
 219 oligosaccharides were determined. This process was facilitated by a comparison with a previously
 220 analyzed tetrasaccharide fraction generated by the Fenton type free radical depolymerisation of DS,
 221 which used H₂O₂ to generate radicals and Cu²⁺ as the catalyst (Panagos, Thomson, Bavington & Uhrin,
 222 2012). This also gave us the opportunity to compare the outcomes of the Fenton type and
 223 photochemical depolymerisation. It became immediately obvious that the spectra of fraction I contain
 224 some additional strong signals. Although the anomeric region of the ¹³C spectrum containing the non-
 225 reducing rings signals was practically identical for samples generated by both methods, new strong
 226 signals (95.12 and 97.50 ppm) were found in the anomeric region of the reducing rings for the
 227 photochemically depolymerized fraction I (Figure 2).

228 These new strong signals belong to the reducing end α- and β-IdoA and their integral intensities have
 229 almost the same total intensity as the reducing end GalNAc signals (Figure 2a, b). For comparison, in the
 230 case of Fenton type depolymerisation the IdoA signals contained less than 10% of the total reducing end
 231 signals. This means that the photochemical depolymerisation does not proceed to fully oxidise the
 232 released reducing end IdoA, as was the case with the Fenton type depolymerisation.

233

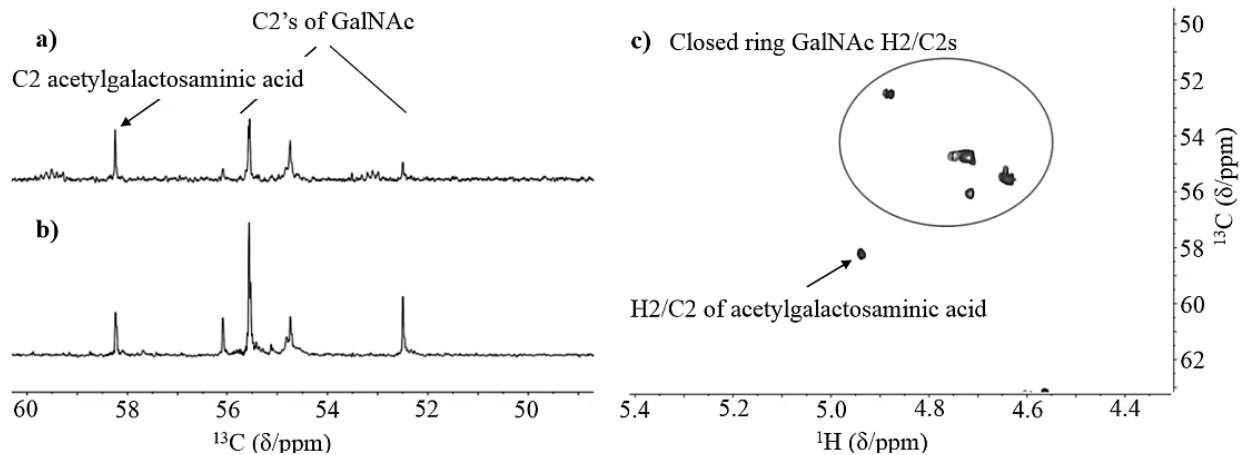


234

235 **Figure 2: Comparison of the reducing end anomeric regions of NMR spectra. 1D ¹³C spectra of tetrasaccharides obtained by**
 236 **(a) photochemical (fraction I) and (b) Fenton type depolymerisation. (c) A partial 2D ¹H, ¹³C HSQC spectrum of fraction I**
 237 **tetrasaccharides obtained by photochemical depolymerisation.**

238 It is now well established, that Fenton type depolymerisation proceeds to partially oxidise the released
 239 reducing end HexNAc as seen in heparin and DS, forming acetylglucosaminic acid and
 240 acetylgalactosaminic acid, respectively (Panagos, Thomson, Bavington & Uhrin, 2012; Vismara, Pierini,
 241 Guglieri, Liverani, Mascellani & Torri, 2007; Vismara et al., 2010) Focusing on the most prominent C2
 242 acetylgalactosaminic acid signal at 58.23 ppm, it is evident (Figure 3) that this signal is stronger in the
 243 photochemically depolymerized sample. Here the acetylgalactosaminic acid accounts for ~22% of the
 244 total reducing end GalNAc signals, whereas in case of Fenton type depolymerisation it was less than

245 13%. It is notable that the analysis of the 2D ^1H ^{13}C HSQC spectrum of the sample showed that there
 246 were no signals corresponding to the open chain reducing end IdoA, which indicates that opening of the
 247 IdoA monosaccharide leads to its complete degradation.



248
 249 **Figure 3: Comparison of the GalNAc C2 region of the NMR spectra of tetrasaccharides obtained by (a) photochemical and (b)**
 250 **Fenton type depolymerisation. (c) A partial 2D ^1H , ^{13}C HSQC spectrum of the H2/C2 region of fraction I.**
 251
 252

253 Finally, a comparison of the integrals of the reducing and non-reducing anomeric ^{13}C signals of fraction I
 254 showed ratio of 1:2.7. If this fraction contained purely tetrasaccharides this should be 1:3. Since the
 255 anomeric carbon of the terminal acetylgalactosaminic acid is oxidised and does not resonate in the
 256 integrated anomeric region, this ratio should be even larger, reflecting the presence of 20% of the open
 257 chain species in fraction I. This apparent discrepancy can be explained by the presence of trisaccharides
 258 in fraction I, as additionally confirmed by the analysis of the MS data (data not shown). At the same time
 259 the IdoA and GalNAc reducing-end signals show intensity ratio of 1:1. It is therefore logical to assume
 260 that both IdoA-GalNAc-IdoA and GalNAc-IdoA-GalNAc trisaccharides are present in fraction I.

261 Photochemical depolymerisation was also performed on fucosylated chondroitin sulfate from *H. forskali*
 262 (fCS) as described previously (Panagos, Thomson, Bavington & Uhrin, 2012). The repeating trisaccharide
 263 unit of this fCS, $\rightarrow 3\text{GalNAc}4,6\text{S}(1\beta\rightarrow 4)[\text{Fuc}\alpha\text{X}1\rightarrow 3]\text{GlcA}(1\beta\rightarrow$, contains branching fucose. The X in this
 264 formula signifies different sulfation patterns of fucose and stands for sulfation at positions 3,4 or 2,4 or
 265 4, which were present at levels of 46%, 39% and 15%, respectively. Other sea cucumber species produce
 266 similar polysaccharides that differ only in the level of sulfation (Vieira & Mourao, 1988; Wu et al., 2013;
 267 Yoshida, Minami, Nemoto, Numata & Yamanaka, 1992). As this polysaccharide is resistant to enzymatic
 268 depolymerisation, it is therefore a good candidate for photochemical depolymerisation. The branched
 269 structure and heterogeneity of the sulfation predisposes the fCS polysaccharide to yield a more complex
 270 mixture of oligosaccharides than the one generated from DS, which prevented their full
 271 characterization. Nevertheless, similar features were observed as for the DS. In particular, the amount of
 272 the N-acetylgalactosaminic acid was significantly increased in smaller fractions, exceeding 60% of the
 273 total reducing end content of GalNAc (data not shown). The oligosaccharides fractions (dp3-dp10) were
 274 used in microarray binding assays and were shown to bind to L- and P-selectins (Panagos et al., 2014).
 275 Since fCS-selectin binding is dependent on the presence of sulfated fucosylated branches, these results

276 indicate that photochemical depolymerisation neither cleaved the fucose branches nor caused
277 desulfation of the polysaccharide.

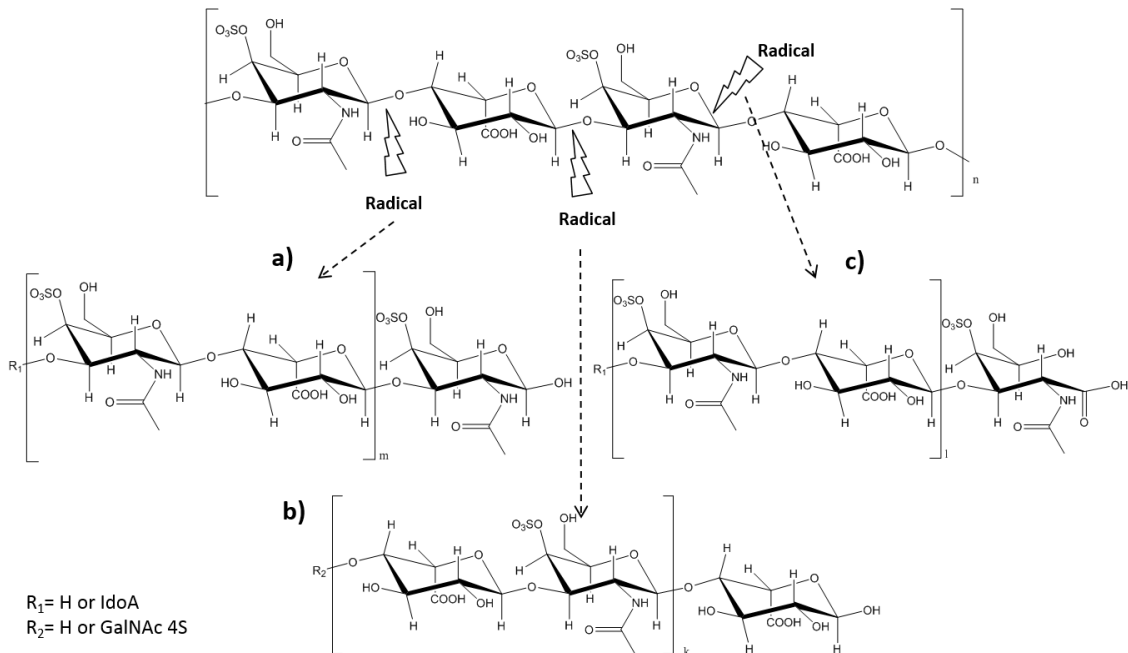
278

279 **4. Discussion and conclusions**

280 Catalytic photochemical depolymerisation of DS was achieved by titanium dioxide in the presence of UV
281 light. In this study we have modified the controlled depolymerisation reaction reported previously
282 (Burana-osot, Hosoyama, Nagamoto, Suzuki, Linhardt & Toida, 2009; Burana-osot,
283 Soonthornchareonnon, Hosoyama, Linhardt & Toida, 2010; Higashi et al., 2012; Higashi et al., 2011), in
284 order to achieve a larger scale (x30 times) and without the use of specialized equipment. A wide range
285 of oligosaccharides, from dp2 to dp22 as shown by SEC, was obtained for DS. These oligosaccharides
286 contained both odd and even numbered species with no desulfation observed.

287 The tetrasaccharide fractions generated by the Fenton type and photochemical depolymerisation of DS
288 were compared (Panagos, Thomson, Bavington & Uhrin, 2012). Despite the fact that both of techniques
289 are based on oxygen free radicals creating random scissions of mainly glycosidic linkages, important
290 structural differences between the two tetrasaccharides were observed. These differences could be
291 caused by possible differences in the free radicals generated through the two techniques. Fenton-type
292 reaction depolymerisation generates almost exclusively hydroxyl radicals, which are known as the most
293 reactive species of the oxygen radical family (Vismara, Pierini, Guglieri, Liverani, Mascellani & Torri,
294 2007). On the other hand excitement of TiO₂ by UV leads to the generation of superoxide radicals. At the
295 same time, the more reactive hydroxyl radicals are produced (Xiang, Yu & Wong, 2011).

296 The most notable difference between the two depolymerisation methods was that the photochemical
297 depolymerisation did not degrade unsulfated IdoA as reported for heparin (Nagasawa, Uchiyama, Sato &
298 Hatano, 1992) and DS (Ofman, Slim, Watt & Yorke, 1997; Panagos, Thomson, Bavington & Uhrin, 2012)
299 depolymerised by Fenton type reaction. Photochemical depolymerisation is, up to now, the only
300 depolymerisation method that preserves reducing end unsulfated IdoA (Figure 4). This opens the
301 possibility of creating a wider range of oligosaccharides that could have novel biological activities.



302

303 **Figure 4: Free radical depolymerisation of a DS polysaccharide, produced oligosaccharides with a) reducing end GalNAc, b)**
 304 **reducing end IdoA and c) reducing end acetylgalactosaminic acid. The non-reducing end terminal monosaccharide can be**
 305 **either IdoA or GalNAc 4S, as indicated. NMR analysis showed no evidence of changes in the structure of non-reducing end**
 306 **monosaccharides after photochemical depolymerisation.**

307 While the photochemical depolymerisation has a milder effect on the reducing end IdoA than the
 308 Fenton type depolymerisation, this is not the case for the reducing end GalNAc. The NMR analysis
 309 showed that the percentage of reducing end GalNAc oxidised to N-acetylgalactosaminic acid increased
 310 compared to the Fenton type depolymerisation. These differences cannot therefore be fully justified by
 311 stating that the photochemical depolymerisation is milder; they rather imply subtle differences in
 312 molecular mechanism between the two methods, possibly due to the differences in the radicals
 313 produced.

314 Since photochemical depolymerisation did not degrade reducing end IdoA, and assuming that it also
 315 cleaves glycosidic linkages randomly, the existence of a trisaccharide in the DS fractions was to be
 316 expected. This was verified by NMR and MS (Panagos, Thomson, Bavington & Uhrin, 2012) and DS
 317 trisaccharides (M_w 653.5 or 760.6 g/mol) were shown to be co-eluted with the tetrasaccharide (M_w
 318 936.7 g/mol) fraction.

319 In summary, this study describes a larger scale photochemical depolymerisation of a simple (DS), which
 320 did not require pH monitoring or specialized equipment. This method generated a range of
 321 oligosaccharides with both odd and even numbers of sugar residues, as previously reported for heparin
 322 (Higashi et al., 2012). Additionally, structural differences were uncovered, by 1D and 2D NMR, between
 323 the oligosaccharides generated by photochemical depolymerisation and Fenton type depolymerisation.
 324 These results indicate that photochemical depolymerisation should be classified as a distinct
 325 depolymerisation technique that enables production of oligosaccharides containing unsulfated reducing-

326 end uronic acid and saturated non-reducing end monosaccharides rings. The photochemical
327 depolymerisation method thus enables preparation of new class of GAG derived oligosaccharides that
328 now can be investigated for their biochemical/biological properties.

329 **Acknowledgements**

330 This work was financially supported by KTP009399 award from the Technology Strategy Board and
331 Scottish Funding Council and GlycoMar Ltd.

332
333 Burana-osot, J., Hosoyama, S., Nagamoto, Y., Suzuki, S., Linhardt, R. J., & Toida, T. (2009). Photolytic
334 depolymerization of alginate. *Carbohydrate Research*, 344(15).
335 Burana-osot, J., Soonthornchareonnon, N., Hosoyama, S., Linhardt, R. J., & Toida, T. (2010). Partial
336 depolymerization of pectin by a photochemical reaction. *Carbohydrate Research*, 345(9).
337 Choay, J., Lormeau, J. C., Petitou, M., Sinaÿ, P., & Fareed, J. (1981). Structural studies on a biologically
338 active hexasaccharide obtained from heparin. *Ann N Y Acad Sci*, 370, 644-649.
339 Comper, W. D., & Laurent, T. C. (1978). Physiological function of connective tissue polysaccharides.
340 *Physiol Rev*, 58(1), 255-315.
341 Dietrich, C. P., Sampaio, L. O., Montesdeoca, H., & Nader, H. B. (1980). Role of sulfated
342 mucopolysaccharides in cell recognition and neoplastic transformatio. *Anais Da Academia Brasileira De*
343 *Ciencias*, 52(1), 179-186.
344 Dol, F., Petitou, M., Lormeau, J. C., Choay, J., Caranobe, C., Sie, P., Saivin, S., Houin, G., & Boneu, B.
345 (1990). Pharmacologic properties of a low molecular weight dermatan sulfate - Comparison with
346 unfractionated dermatan sulfate. *Journal of Laboratory and Clinical Medicine*, 115(1).
347 Gallagher, J. T. (2006). Multiprotein signalling complexes: regional assembly on heparan sulphate.
348 *Biochem Soc Trans*, 34(Pt 3), 438-441.
349 Gray, E., Mulloy, B., & Barrowcliffel, T. W. (2008). Heparin and low-molecular-weight heparin.
350 *Thrombosis and Haemostasis*, 99(5).
351 Guerrini, M., Beccati, D., Shriver, Z., Naggi, A., Viswanathan, K., Bisio, A., Capila, I., Lansing, J. C., Guglieri,
352 S., Fraser, B., Al-Hakim, A., Gunay, N. S., Zhang, Z., Robinson, L., Buhse, L., Nasr, M., Woodcock, J.,
353 Langer, R., Venkataraman, G., Linhardt, R. J., Casu, B., Torri, G., & Sasisekharan, R. (2008). Oversulfated
354 chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nat Biotechnol*,
355 26(6), 669-675.
356 Higashi, K., Hosoyama, S., Ohno, A., Masuko, S., Yang, B., Sterner, E., Wang, Z., Linhardt, R. J., & Toida, T.
357 (2012). Photochemical preparation of a novel low molecular weight heparin. *Carbohydrate Polymers*,
358 87(2), 1737-1743.
359 Higashi, K., Ly, M., Wang, Z., Masuko, S., Bhaskar, U., Sterner, E., Zhang, F., Toida, T., Dordick, J. S., &
360 Linhardt, R. J. (2011). Controlled photochemical depolymerization of K5 heparosan, a bioengineered
361 heparin precursor. *Carbohydrate Polymers*, 86(3).
362 Hileman, R. E., Smith, A. E., Toida, T., & Linhardt, R. J. (1997). Preparation and structure of heparin lyase-
363 derived heparan sulfate oligosaccharides. *Glycobiology*, 7(2), 231-239.
364 Legnani, C., Palareti, G., Biagi, R., Ludovici, S., Maggiore, L., Milani, M. R., & Coccheri, S. (1994). Acute
365 and chronic effects of a new low molecular weight dermatan sulphate (Desmin 370) on blood
366 coagulation and fibrinolysis in healthy subjects. *Eur J Clin Pharmacol*, 47(3), 247-252.
367 Mathews, M. B. (1975). Connective tissue. Macromolecular structure and evolution. *Mol Biol Biochem*
368 *Biophys*(19), 1-318.

- 369 Matzsch, T., Bergqvist, D., Hedner, U., Nilsson, B., & Ostergaard, P. (1990). Effects of low molecular
370 weight heparin and unfragmented heparin on induction of osteoporosis in rats. *Thrombosis and*
371 *Haemostasis*, 63(3).
- 372 Medeiros, G. F., Mendes, A., Castro, R. A., Baú, E. C., Nader, H. B., & Dietrich, C. P. (2000). Distribution of
373 sulfated glycosaminoglycans in the animal kingdom: widespread occurrence of heparin-like compounds
374 in invertebrates. *Biochim Biophys Acta*, 1475(3), 287-294.
- 375 Miyazaki, T., Yomota, C., & Okada, S. (2001). Ultrasonic depolymerization of hyaluronic acid. *Polymer*
376 *Degradation and Stability*, 74(1).
- 377 Monreal, M., Vinas, L., Monreal, L., Lavin, S., Lafoz, E., & Angles, A. M. (1990). Heparin-related
378 osteoporosis in rats. A comparative study between unfractionated heparin and a low-molecular-weight
379 heparin. *Haemostasis*, 20(4).
- 380 Mourao, P. A. S., Pereira, M. S., Pavao, M. S. G., Mulloy, B., Tollefsen, D. M., Mowinckel, M. C., &
381 Abildgaard, U. (1996). Structure and anticoagulant activity of a fucosylated chondroitin sulfate from
382 echinoderm - Sulfated fucose branches on the polysaccharide account for its high anticoagulant action.
383 *Journal of Biological Chemistry*, 271(39), 23973-23984.
- 384 Munoz, E. M., & Linhardt, R. J. (2004). Heparin-binding domains in vascular biology. *Arteriosclerosis*
385 *Thrombosis and Vascular Biology*, 24(9), 1549-1557.
- 386 Nagasawa, K., Uchiyama, H., Sato, N., & Hatano, A. (1992). Chemical change involved in the oxidative-
387 reductive depolymerization of heparin. *Carbohydr Res*, 236, 165-180.
- 388 Ofman, D., Slim, G. C., Watt, D. K., & Yorke, S. C. (1997). Free radical induced oxidative depolymerisation
389 of chondroitin sulphate and dermatan sulphate. *Carbohydrate Polymers*, 33(1), 47-56.
- 390 Panagos, C., Thomson, D., Bavington, C. D., & Uhrin, D. (2012). Structural characterisation of
391 oligosaccharides obtained by Fenton-type radical depolymerisation of dermatan sulfate. *Carbohydrate*
392 *Polymers*, 87(3), 2086-2092.
- 393 Panagos, C., Thomson, D. S., Moss, C., Hughes, A. D., Kelly, M. S., Liu, Y., Chai, W., Venkatasamy, R.,
394 Spina, D., Page, C. P., Hogwood, J., Woods, R. J., Mulloy, B., Bavington, C. D., & Uhrin, D. (2014).
395 Fucosylated chondroitin sulfates from the body wall of the sea cucumber *Holothuria forskali*.
396 Conformation, selectin binding and biological activity. *J Biol Chem*. 2014;289(41):28284-28298.
- 397 Pervin, A., Gallo, C., Jandik, K. A., Han, X. J., & Linhardt, R. J. (1995). Preparation and structural
398 characterization of large heparin-derived oligosaccharides. *Glycobiology*, 5(1), 83-95.
- 399 Singer, S. J. (1992). Intercellular communication and cell-cell adhesion. *Science*, 255(5052), 1671-1677.
- 400 Suzuki, N., Kitazato, K., Takamatsu, J., & Saito, H. (1991). Antithrombotic and anticoagulant activity of
401 depolymerized fragment of the glycosaminoglycan extracted from *Stichopus japonicus* Selenka. *Thromb*
402 *Haemost*, 65(4), 369-373.
- 403 Toida, T., Sato, K., Sakamoto, N., Sakai, S., Hosoyama, S., & Linhardt, R. J. (2009). Solvolytic
404 depolymerization of chondroitin and dermatan sulfates. *Carbohydrate Research*, 344(7).
- 405 Uchiyama, H., Dobashi, Y., Ohkouchi, K., & Nagasawa, K. (1990). Chemical change involved in the
406 oxidative reductive depolymerization of hyaluronic acid. *J Biol Chem*, 265(14), 7753-7759.
- 407 Vieira, R. P., & Mourao, P. A. S. (1988). Occurrence of a unique fucose-branched chondroitin sulfate in
408 the body wall of a sea cucumber. *Journal of Biological Chemistry*, 263(34), 18176-18183.
- 409 Vieira, R. P., Mulloy, B., & Mourao, P. A. S. (1991). Structure of a fucose-branched chondroitin sulfate
410 from sea cucumber. Evidence for the presence of 3-O-sulfo-beta-D-glucuronosyl residues. *Journal of*
411 *Biological Chemistry*, 266(21), 13530-13536.
- 412 Vismara, E., Pierini, M., Guglieri, S., Liverani, L., Mascellani, G., & Torri, G. (2007). Structural modification
413 induced in heparin by a Fenton-type depolymerization process. *Semin Thromb Hemost*, 33(5), 466-477.
- 414 Vismara, E., Pierini, M., Mascellani, G., Liverani, L., Lima, M., Guerrini, M., & Torri, G. (2010). Low-
415 molecular-weight heparin from Cu²⁺ and Fe²⁺ Fenton type depolymerisation processes. *Thromb*
416 *Haemost*, 103(3), 613-622.

- 417 Vreys, V., & David, G. (2007). Mammalian heparanase: what is the message? *Journal of Cellular and*
418 *Molecular Medicine*, 11(3), 427-452.
- 419 Wu, M., Xua, S., Zhao, J., Kang, H., & Ding, H. (2010). Physicochemical characteristics and anticoagulant
420 activities of low molecular weight fractions by free-radical depolymerization of a fucosylated chondroitin
421 sulphate from sea cucumber *Thelenata ananas*. *Food Chemistry*, 122(3), 716-723.
- 422 Wu, M. Y., Xu, S. M., Zhao, J. H., Kang, H., & Ding, H. (2010a). Free-radical depolymerization of
423 glycosaminoglycan from sea cucumber *Thelenata ananas* by hydrogen peroxide and copper ions.
424 *Carbohydrate Polymers*, 80(4), 1116-1124.
- 425 Wu, M. Y., Xu, S. M., Zhao, J. H., Kang, H., & Ding, H. (2010b). Preparation and characterization of
426 molecular weight fractions of glycosaminoglycan from sea cucumber *Thelenata ananas* using free radical
427 depolymerization. *Carbohydrate Research*, 345(5), 649-655.
- 428 Wu, N., Ye, X., Guo, X., Liao, N., Yin, X., Hu, Y., Sun, Y., Liu, D., & Chen, S. (2013). Depolymerization of
429 fucosylated chondroitin sulfate from sea cucumber, *Pearsonothuria graeffei*, via Co-60 irradiation.
430 *Carbohydrate Polymers*, 93(2), 604-614.
- 431 Xiang, Q., Yu, J., & Wong, P. K. (2011). Quantitative characterization of hydroxyl radicals produced by
432 various photocatalysts. *J Colloid Interface Sci* (Vol. 357, pp. 163-167). United States: 2011 Elsevier Inc.
- 433 Yang, J., Wang, Y., Jiang, T., Lv, L., Zhang, B., & Lv, Z. (2015). Depolymerized glycosaminoglycan and its
434 anticoagulant activities from sea cucumber *Apostichopus japonicus*. *Int J Biol Macromol*, 72, 699-705.
- 435 Yoshida, K., Minami, Y., Nemoto, H., Numata, K., & Yamanaka, E. (1992). Structure of DHG, a
436 depolymerized glycosaminoglycan from sea cucumber, *Stichopus japonicus*. *Tetrahedron Letters*, 33(34),
437 4959-4962.