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Mass spectrometric evidence for the mechanism of free-radical depolymerization of various types of glycosaminoglycans

Ning Zhang[a], Guoyun Li[a,b,*], Shijie Li[a], Chao Cai[a,b], Fuming Zhang[c], Robert J. Linhardt[c], Guangli Yu[a,b]

[a] Key Laboratory of Marine Drugs, Ministry of Education, School of Medicine and Pharmacy, Shandong Provincial Key Laboratory of Glycoscience and Glycotechnology, Ocean University of China, Qingdao, 266003, China
[b] Laboratory for Marine Drugs and Bioproducts, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao, 266003, China
[c] Department of Chemistry and Chemical Biology, Biomedical Engineering, Biology, Chemical and Biological Engineering, and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

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ABSTRACT

Glycosaminoglycans (GAGs) are large, complex carbohydrate molecules that interact with a wide range of proteins involved in physiological and pathological processes. Several naturally derived GAGs have emerged as potentially useful therapeutics in clinical applications. Natural polysaccharides, however, generally have high molecular weights with a degree of polydispersity, making it difficult to investigate their structural properties. In this study, we establish a free-radical–mediated micro-reaction system and use hydrophilic interaction chromatography (HILIC)–Fourier transform mass spectrometry (FTMS) to profile the degraded products of various types of GAGs, heparin, chondroitin sulfate A, NS-heparosan, and oversulfated chondroitin sulfate (OSCS), to reveal the free-radical degradation mechanism of GAGs. The results show that the bulk fragments of GAGs generated by free-radical degradation can maintain their basic structural units and sulfate substituents. In addition, an abundance of oligomers modified with oxidation at their reducing ends or by dehydration also appeared. We discovered that these modifications were related in terms of the degree of sulfation and the α- or β-linkage of HexNY (Y = SO_3^- or Ac), and especially that the different linkage of the disaccharide unit is the main factor in modification. In addition, the method based on micro-free-radical reaction and HILIC-FTMS is both effective and sensitive, thus suggesting its broad practical value for the structural characterization and in the biological structure-function studies of GAGs.

1. Introduction

Glycosaminoglycans are a family of linear, sulfated, negatively charged polysaccharides that have molecular weights of roughly 10–100 kDa (Booth & Thomason, 1991; Gandhi & Mancera, 2008). Based on differences among the repeating disaccharide units comprising GAGs, they can be categorized into four main groups: chondroitin sulfate (CS)/dermatan sulfate (DS), heparin (HP)/heparan sulfate (HS), hyaluronan (HA), and keratan sulfate (KS). *In vivo*, most GAGs are linked to the core protein as proteoglycans (PGs), while HA exists only as a GAG. The characterization and identification of GAGs in biological samples can reveal the relationship between their structures and their biological functions (Chen et al., 2013; Li, Ly, & Linhardt, 2012). In addition to their physiological and pathophysiological roles, these naturally complex polysaccharides are active biological and pharmaceutical agents (Li, Ly et al., 2012; Silva et al., 2019). For this reason, several GAG-based drugs are already in clinical use. For example, the naturally occurring anticoagulant heparin is a heterogeneous, polydisperse, highly sulfated polysaccharide belonging to the GAG family. The low-molecular weight heparins (LMWHs) have become the drugs of choice for the treatment of deep vein thrombosis, pulmonary embolism, arterial thrombosis, and unstable angina (Quader, Stump, & Sumpio, 1998), and they have been shown to improve the survival of cancer patients (Furukawa, Okada, & Shinohara, 2017; Green, Hull, Brant, & Pineo, 1992). In addition, the *EULAR Recommendations for the Treatment of Knee OA*, published in 2003, listed oral CS as both evidence 1A and strength of recommendation A, representing the highest level for a therapeutic strategy (Jordan et al.,

* Corresponding author at: Key Laboratory of Marine Drugs, Ministry of Education, School of Medicine and Pharmacy, Shandong Provincial Key Laboratory of Glycoscience and Glycotechnology, Ocean University of China, Qingdao, 266003, China.
E-mail address: liguoyun@ouc.edu.cn (G. Li).

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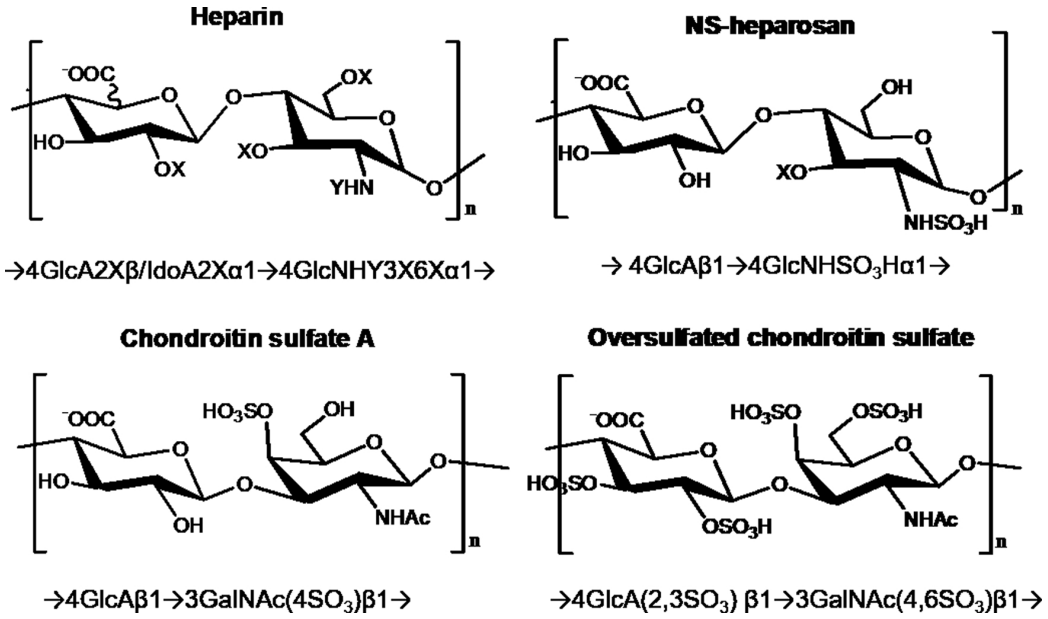

Fig. 1. Structures of 4 types of GAGs.

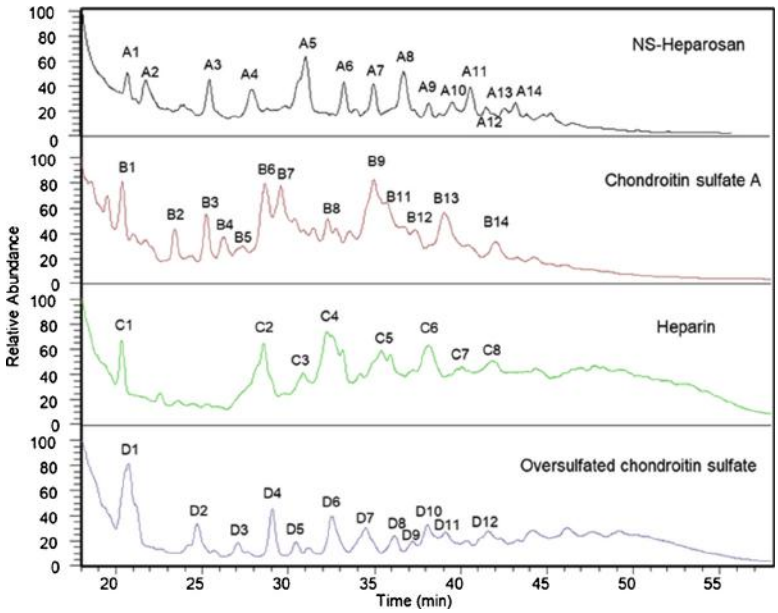

Fig. 2. Total ion chromatograms of four types of free radical depolymerized GAGs.

2003; Richy et al., 2003; Uebelhart, 2008). Currently, GAGs have a wide range of medical applications, from topical moisturizers to anticoagulants used in most surgical procedures, and exhibit great pharmaceutical value (DeAngelis, 2012; Yip, Smollich, & Gotte, 2006).

The fundamental biological, pathological, pharmacological, and therapeutic roles of GAGs have challenged researchers to devise new processes to prepare these critical polysaccharides, as well as novel methods to decode their fine complex structures, procedures that are needed to establish their structure-activity relationships. The direct analysis of intact GAGs is difficult due to their relatively high molecular mass and polydispersity. As a consequence, controlled enzymatic or chemical depolymerization is often required to lower their molecular weights and simplify their polydisperse mixtures prior to analysis. Natural GAGs are high-molecular-weight polymers with complex structures. Only a few types of GAGs have specific cleavage enzymes, and if their structural or chemical groups change, the original enzymes will lose their specific effect on the modified GAGs. Therefore, it is desirable to establish a widely applicable GAG degradation method. Chemical degradation methods include acid or alkali degradation, oxidant degradation, free-radical degradation, and others. Acid or alkali degradation is difficult to control and often results in damage to GAG structure. Different preparation methods result in products that differ from each other structurally. One of the most commonly used processes of GAG chemical depolymerization is the process carried out by free-radical attack (mainly hydroxyl). In recent years, reactive oxygen species (ROS) produced by chemical agents or radiation has been used to

Table 1
Assignments of the chromatographic peaks of degraded GAGs analyzed using HILIC-FTMS.

	A NS-heparosan	B CSA	C Heparin	D OSCS
Peak 1	(1,1,0,1)	(1,1,1,1)	(1,1,0,2) (1,1,0,3)	(1,1,1,4)
Peak 2	(1,1,0,1)	(1,1,1,1) (1,2,2,2) (1,1,1,1)-A*	(1,2,0,5)	(1,2,2,5) (1,2,2,6)
Peak 3	(1,2,0,2)	(2,2,2,0)	(1,2,0,5)-A	(1,1,1,4)-A
Peak 4	(2,1,0,1)	(2,2,2,0)	(2,2,0,4) (2,2,0,5) (2,2,0,6) (2,2,0,7)	(1,2,2,4)-A (1,2,2,6)-A
Peak 5	(2,2,0,2)	(1,2,2,2)-A	(2,3,0,7) (2,3,0,8) (2,3,0,5) (3,2,0,6)	(1,2,1,5)-A (2,1,1,5) (2,1,1,6)
Peak 6	(2,3,0,3)	(2,2,2,2)	(3,3,0,7) (3,3,0,8) (3,3,0,9)	(2,2,2,7) (2,2,2,8)
Peak 7	(3,2,0,2)	(2,3,3,3)	(3,3,0,8)-A (3,4,0,11)	(2,2,1,7)-A (2,2,2,7)-A (2,2,2,9) (2,3,3,9) (2,3,3,10) (3,3,0,9) (3,3,2,10)
Peak 8	(3,3,0,3)	(2,2,2,2)-A (3,2,2,0)	(4,4,0,11) (4,4,0,12)	(2,3,3,10)-A (3,3,3,8)-A
Peak 9	(3,4,0,4)	(3,2,2,1) (3,3,3,1)	(5,4,0,13)	(2,3,1,11)-A (2,3,3,9) (3,2,2,10)
Peak 10	(4,3,0,3)	(3,2,2,2) (3,3,3,3)	—	(3,3,3,12)
Peak 11	(4,4,0,4)	(3,2,2,2)-A (3,4,4,4)	—	(3,4,4,14)
Peak 12	(4,5,0,5)	(3,3,3,3)-A	—	(4,3,3,14)
Peak 13	(5,4,0,4)	(4,3,3,3)-A (4,4,4,4)	—	—
Peak 14	—**	(5,4,4,4)	—	—

* The (a, b, c, d) in table represents the composition of HexA, HexN, Ac (acetyl), and SO_3 (sulfate groups) in oligosaccharide components, respectively. The A means the carboxylic acids in reducing ends.

** Non-assignment.

degrade sulfated polysaccharides (Kjellen & Lindahl, 1991; Ofman, Slim, Watt, & Yorke, 1997; Vismara et al., 2010; Zhao, Yang, Li, Zhang, & Linhardt, 2013). Free-radical degradation, which is based on the Fenton reaction and is a common chemical degradation method in GAG analysis, differs from enzymatic degradation and can be applied to all polysaccharides without selectivity. Vismara et al. (2007), (2010) has described a mechanism for this reaction. With the catalysis of transition metal ions (Cu^+, Fe^{2+}, and others), H_2O_2 decomposes to produce active ˙OH, which can quickly attack the glycan chain and capture the H connected to the carbon to form a hydroxyalkyl radical. The ˙C-O can also be oxidized by Cu^{2+} or Fe^{3+} to +C-O, and finally, the +C-O will be converted into ketone, aldehyde, or acid based on the reaction conditions or the reaction medium. Free-radical degradation of various GAGs can maintain the original structural information, does not cause branch breakage or loss of sulfate groups, is highly reproducible, is inexpensive, and, therefore, is widely used. However, free-radical degradation is a complex process that results in byproducts. Thus, the development of suitable analytical methods is challenging.

Structural analysis of the various domains of GAGs is necessary in order to determine their biological activities, but these experiments have been limited by the extreme heterogeneity of GAGs. Mass spectrometry (MS) is now emerging as the method of choice for GAG oligosaccharide analysis (Galeotti & Volpi, 2016; Saad et al., 2005; Sanderson et al., 2018; Shi & Zaia, 2009). Linhardt and coworkers (Ly et al., 2011) first determined the structural sequence of GAGs in bikunin based on a top-down method using FTMS and FT-ICR-MS. Capillary amide hydrophilic interaction chromatography (HILIC) with online electrospray ionization (ESI) MS and high-performance liquid chromatography (HPLC) has been used for the analysis of released glycans (Wuhrer, Koeleman, Deelder, & Hokke, 2004), glycopeptides (Merry et al., 2002; van Schaick, Pirok, Haselberg, Somsen, & Gargano, 2019), glycosphingolipids (Wing et al., 2001), and GAGs (Staples et al., 2010). Li, Zhang, Zaia, and Linhardt, (2012)) used HILIC-FTMS to analyze enoxaparin sodium and obtained more than 300 pieces of glycan-related data, including non-reducing end components, glycans with or without unsaturated bonds, and glycans containing 1, 6-anhydro derivatives at their reducing ends. Although the combination of online high-performance chromatography and mass spectrometry provides an efficient technical basis for structural and compositional analysis of GAGs, specialized analytical techniques and bioinformatics are also necessary. Therefore, we utilized the publicly available open source GlycReSoft software developed by Zaia and coworkers (Maxwell et al., 2012) to calculate bioinformatics search space sets for GAG samples and to assign oligosaccharides.

In this study, HILIC in combination with high-resolution FTMS were applied for the identification and quantification of the GAG-derived oligosaccharides generated by free-radical degradation. By analyzing the structural characteristics of the products, the structural quantification of GAG-derived oligosaccharides was established, which provides a critical advantage for revealing the reaction mechanism of different GAGs in free-radical degradation. This finding was also further used to study the structure-activity relationships and potential pharmaceutical values of GAGs.

2. Experiments

2.1. Materials

Heparan sulfate (CID 53477715 - National Center for Biotechnology Information, PubChem Compound Database) and heparin (CID 772 - National Center for Biotechnology Information, PubChem Compound Database) were purchased from Celsus (Cincinnati, Ohio, USA); NS-heparosan (Bhaskar et al., 2015) and OSCS (B. Li et al., 2009) were prepared in our laboratory. CSA (CID 4368136 - National Center for Biotechnology Information, PubChem Compound Database), H_2O_2, cupric acetate monohydrate, acetonitrile (chromatography grade), ammonium acetate (chromatography grade), and other experimental reagents were purchased from Sigma–Aldrich® (St. Louis, MO, USA). The average molecular (Mw) of heparin, NS-heparosan, CSA and OSCS is 24.9 kDa, 18.8 kDa, 24.2 kDa and 18.0 kDa. The 1200 high-performance liquid chromatography system was purchased from Agilent Technologies (Wilmington, DE, USA), the LTQ Orbitrap XL mass spectrometer was purchased from Thermo Fisher Scientific (Waltham, MA, USA), and the Luna HILIC Column (2.0 × 150 mm, 200 Å) was purchased from Phenomenex (Torrance, CA, USA).

2.2. Free-radical depolymerization

Different types of GAGs, including heparin, NS-heparosan, CSA, and OSCS, were depolymerized in the micro-free-radical reaction system (Vismara et al., 2010). In Fenton-type reactions, hydrogen peroxide is decomposed in a water solution in the presence of a catalytic amount of a transition metal of low oxidation number. Desalted GAG (100 μg) was dissolved in 100 μL, 50 mM, pH 7.0 sodium acetate-acetate buffer (containing 0.2 mM cupric acetate monohydrate). Next, 4 μL 3 % hydrogen peroxide solution was added to the reaction system, well mixed, and reacted at 45 °C for 3 h. Then the reaction solution was freeze-dried repeatedly to remove excess hydrogen peroxide. Lyophilized samples were dissolved in 50 μL water-acetonitrile solution (V: V, 1:1), centrifuged, and the supernatant was retained for LC–MS analysis.

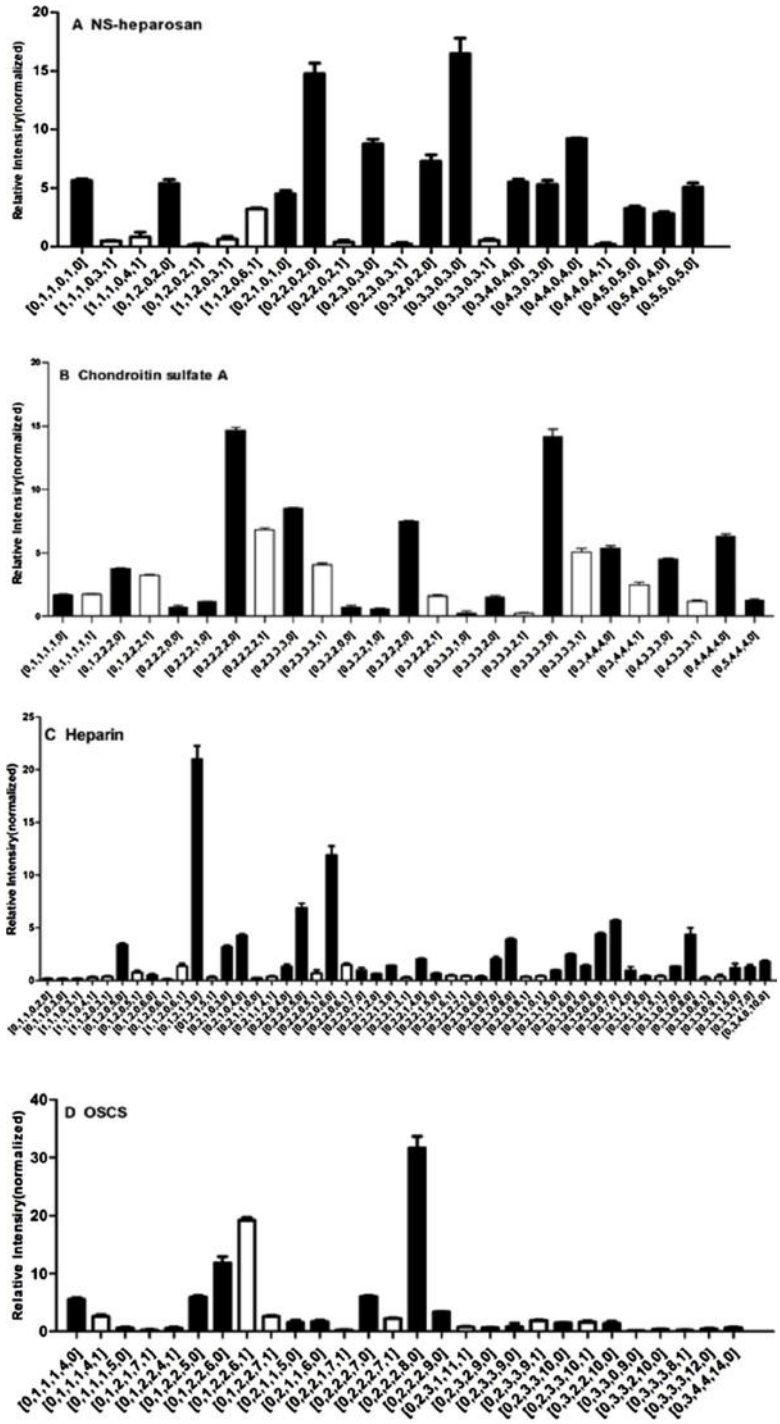

Fig. 3. Quantitative analysis of the GAG-derived oligosaccharides generated by free-radical degradation: A. NS-heparosan, B. CSA, C. Heparin, D. OSCS).

2.3. HILIC-FTMS analysis

Free-radical degraded oligosaccharides were analyzed using HILIC-FTMS, which was performed on an Agilent 1290 LC UPLC system (Agilent Technologies, Wilmington, DE, USA) equipped with an LTQ ORBITRAP XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The oligosaccharides were separated by a Luna HILIC column (150 × 2.00 mm, 3 μm; Phenomenex, Torrance, CA, USA) at 25 °C and the eluted fractions were passed directly to the mass spectrometry system using an ESI source. The mobile phase A was 5 mM ammonium acetate aqueous solution, and the mobile phase B was 5 mM ammonium acetate aqueous solution (containing 98 % acetonitrile). The gradient was programmed as 92 % A at the beginning, linearly changing to 60 % A in 58 min. The flow rate was 150 μL/min. In order to ionize and prevent oligosaccharide molecules from degrading within the ion source, the FTMS detector was in negative ion mode, the spray

Table 2
Oxidation and intramolecular dehydration rates of free-radical degraded products.

	NS-heparosan	CSA	Heparin	OSCS
Oxidation rate (%)	4.36 ± 0.28	12.60 ± 0.19	3.63 ± 0.54	19.20 ± 0.28
Dehydration rate (%)	3.42 ± 0.13	—	1.33 ± 0.22	—

voltage was 4.2 KV, the capillary voltage was −40 V, the capillary temperature was 275 °C, and the tube lens voltage was −50 V. The *m/z* range was 300–2,000, the resolution was 60,000, and the mass spectral deviation was < 3 ppm.

2.4. Bioinformatics software analysis

The LC-FTMS data were acquired and quantified automatically using Decon2LS (Jaitly et al., 2009) (from Internet resources http://omics.pnlgov) and GlycReSoft software. The charge deconvolution was automatically processed using Decon2LS software, and the results were then auto-processed using GlycReSoft software. The GlycReSoft parameters were set as follows: Minimum Abundance: 1.0; Minimum Number of Scans: 1; Molecular Weight Lower Boundary: 200 Da; Molecular Weight Upper Boundary: 6,000 Da; Mass Shift: NH_3; Match Error: 5.0 ppm; Grouping Error: 80 ppm; and Adduct Tolerance: 5.0 ppm. The hypothesis generator parameters were set according to the characteristics of heparin composition. The normalization was based on the abundance of all of the oligosaccharide components after ownership.

3. Results and discussion

3.1. Free-radical depolymerization of different types of GAGs

Compared to enzymatic degradation, the chemical degradation of polysaccharides has a wide range of applications due to its low cost. Chemical degradation methods include acid or alkali degradation, oxidant degradation, and free-radical degradation. In acid or alkali degradation, the reaction conditions are difficult to control, often leading to structural damage to the GAGs. Free-radical degradation based on the Fenton reaction is the most common chemical degradation method employed in the structural analysis of GAGs. It has been shown that free-radical degradation is widely used because it retains the original structural information of GAGs and does not cause branch damage or loss of sulfate groups.

In this study, we established a free-radical degradation micro-reaction system of GAGs, with 10–100 μg samples in a system volume of 100 μL, to which Fenton reagent was added in order to trigger the process of free-radical degradation. After termination of the reaction, the sample was freeze-dried and reconstituted for analysis. Based on the above method, NS-heparosan (the basic skeleton of heparin synthesis), chondroitin sulfate A (extracted from whale cartilage), heparin, and oversulfated chondroitin sulfate (OSCS, fully-sulfated GAGs synthesized chemically by chondroitin sulfate A) were degraded by the free-radical micro-reaction systems and analyzed using HILIC-FTMS. The structures of the four GAGs analyzed are shown in Fig. 1.

3.2. Chromatographic separation of different GAG-derived oligosaccharides on a HILIC column

The mixed oligosaccharides were analyzed using HILIC-FTMS. The total ion chromatograms of the four types of depolymerized GAGs are shown in Fig. 2. As we have reported previously (Li et al., 2014), these oligosaccharides are eluted based on oligomer size. The oligosaccharides of NS-heparosan and OSCS are easier to separate and have a better peak shape mainly due to the homogeneity of NS-heparosan and OSCS, while the multiple peaks in the TIC of the heterogeneous CSA and heparin are clustered together and are more difficult to separate. Heparin and CSA extracted from biological samples are quite inhomogeneous, and heparin chains in particular have different sulfated modifications. More than 70 % of CSA is →4GlcAβ1→3GalNAc ($4SO_3$) β1→but there are also some unsulfated domains. Based on the mass spectrometry data, the chromatographic peaks were assigned and are summarized in Table 1. The composition of the oligosaccharide components in Table 1 is represented by the letters A, B, C, and D, which correspond to the numbers of HexA (hexuronic acid), HexN (hexosamine), Ac (acetyl), and SO_3 (sulfate groups), respectively. Some of the oligosaccharide reducing ends were oxidized to carboxylic acids, so an "A" was appended to their compositions. The pairs A1 and A2, B3 and B4 each had the same composition and are mutually respective isomers. Their structural difference is the sequence order. Similarly, peak A5 (dp4) consists of 2 incompletely separated peaks. Therefore, HILIC columns can resolve the low degree of isomers. However, no isomer peaks were found in either OSCS or heparin, and all of their components were in 1 peak. This also suggests that the differences between these would be eliminated by high sulfation modification.

In the HILIC-FTMS ion chromatogram of heparin, the distribution of oligosaccharides is mainly clustered based on their degree of polymerization. Although the degree of sulfation varies widely, there is no significant effect on the retention of oligosaccharides in HILIC columns. However, the retention times of oxidized oligosaccharides differ from those of non-oxidized oligosaccharides. For example, the chromatographic peak pairs (shown in Fig. 2) D1 (1,1,1,4) and D3 (1,1,1,4)-A, D2 (1,2,2,6) and D4 (1,2,2,6)-A suggest that carboxyl modifications can significantly enhance the retention of oligosaccharides in a HILIC column, and their effects are much greater than those of sulfate modification.

3.3. The basic structural unit of products can remain well preserved, and sulfation modification can resist free-radical degradation

Using the glycobioinformatics software Decon2LS and GlycReSoft, the original data from HILIC-FTMS were automatically analyzed. Quantitative information on the reaction mixture samples is displayed in Fig. 3. Highly sulfated oligosaccharides in the ion source will emerge with partial desulfurization (Leijdekkers, Sanders, Schols, & Gruppen, 2011). As the results reveal, the structure of GAGs can remain well preserved after free-radical degradation. The quantitative result of each GAG effectively reflects its structural features and therefore can be used for the profiling analysis of novel GAGs However, an attack on the backbone of a large-molecular-weight GAG by ˙OH is different from a simple hydrolytic reaction. As we mentioned in the previous paper, the concentration of ˙OH in the reaction is the pivotal factor which effect the degradation degree significantly (Li et al., 2014). According to the molecular weight distribution of the products in the total ion chromatogram, most of the NS-heparosan and CSA with low levels of sulfation are converted to lower molecular weight oligosaccharides, while there are still a large number of higher molecular weight components in OSCS and heparin with higher levels of sulfation. These results indicate that highly sulfated sequences resist the degradation of free radicals. This may be due to the fact that the electron-attraction effect of the sulfate group as well as steric hindrance make it difficult for · OH to acquire H radicals on C to ˙CO radicals and initiate a free-radical chain reaction.

The composition of the oligosaccharide components is [Dehydrated-HexA, HexA, HexN, Ac, SO_3, Carboxyl]; components less than 0.1 % are not included. The black columns represent the oligosaccharides with non-oxidized reducing ends, and the white columns represent the oligosaccharides with oxidized reducing ends.

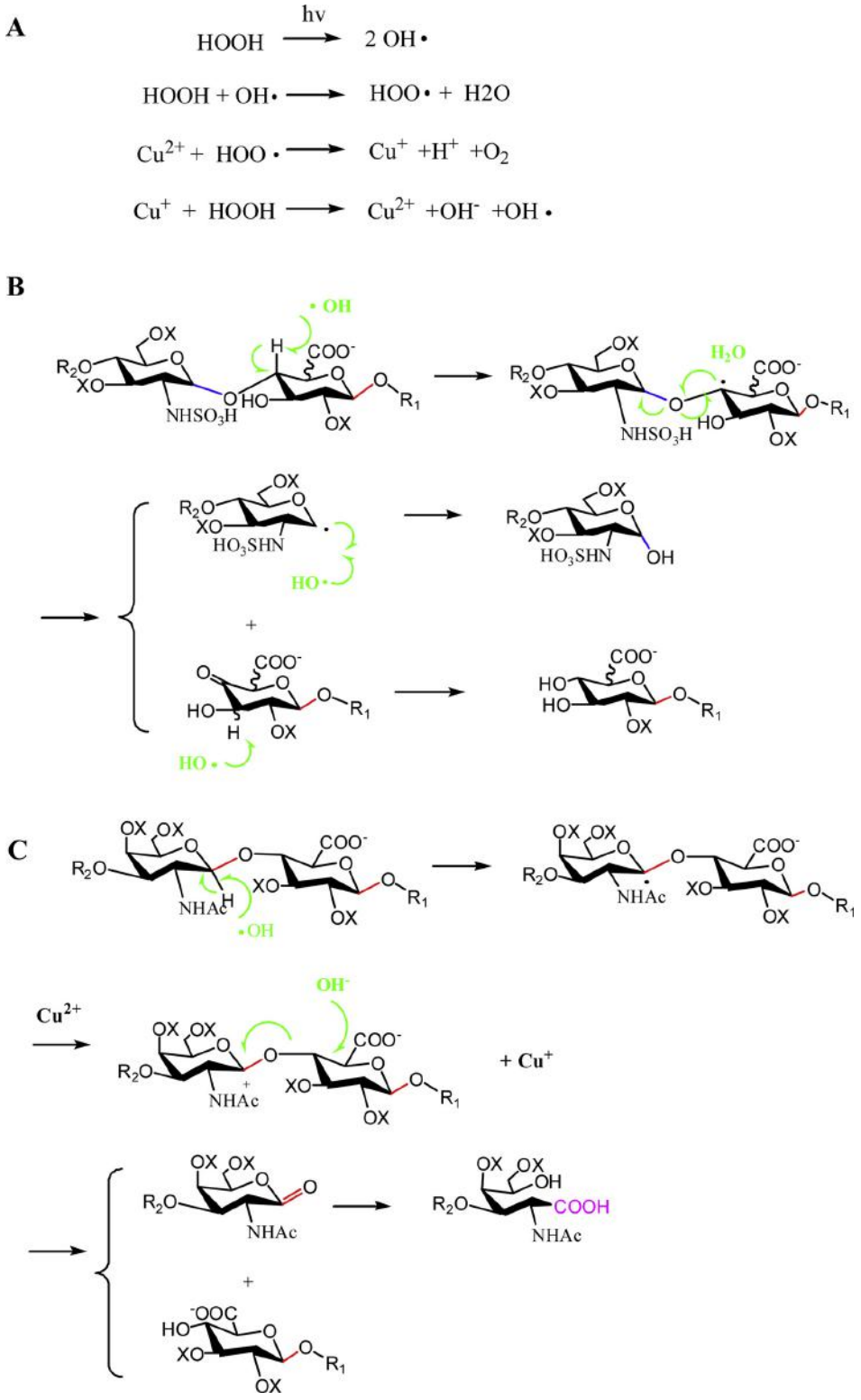

Fig. 4. Proposed mechanism for Fenton-type free radical degradation of different GAGs (A. Free radical degradation of GAGs produce most oligosaccharide maintain their basic structural unit. B. Free radical degradation of GAGs (especially the GAGs with β-linked structural units) involves oxidative breaking of glycosidic bonds).

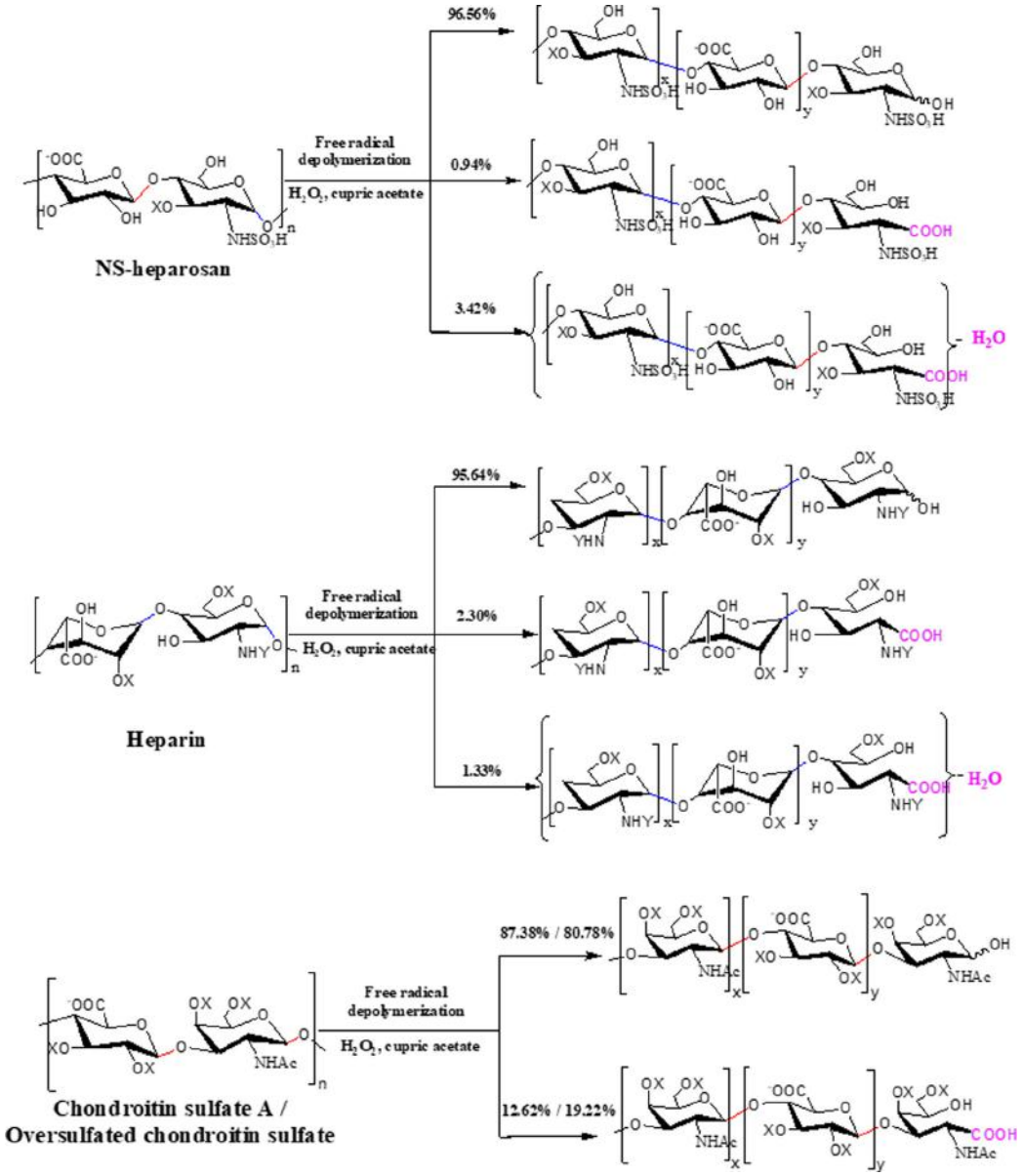

Fig. 5. The different components of GAG-derived oligosaccharides generated by free-radical degradation(x = 0/1, y = 1, 2, 3…). The red link represents β-link, and blue link represents α-link (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.4. The linkage of HexNHY and the degree of sulfation affect the structure of products

The uronic acid residue in heparin has been reported to be the most susceptible site attacked by the radicals produced in the Fenton process (Zhi et al., 2019). As Vismara et al. (2007) found in a free-radical degradation of heparin, in the ion chromatograms of products from CSA, OSCS, and heparin, significant chromatographic peaks are identified as oligosaccharide components with aldonic acid terminals (Table 2 and Fig. S1). The effects of different reaction conditions including the concentration of hydrogen peroxide, reaction time and temperature are compared and investigated. Besides, the concentration of hydrogen peroxide and reaction temperature can both significantly affect the oxidized degree of oligosaccharide (Figs. S2 and S3). As the concentration of hydrogen peroxide and reaction temperature increase, the generated oxidized GAG oligosaccharides are also increased. While reaction time plays a relatively weak role on the degree of oxidation. The related data are shown in the supplementary information.

We further systematically compared the different factors, including the linkage (α or β), level of sulfation, and N-substituent group to explore the effect of carbohydrate-chain structure on free-radical degradation. Compared with their low-sulfation structures, heparin (compared to NS-heparosan) and OSCS (compared to CSA) clearly exhibit a higher degree of oxidation. The level of sulfation in heparin is higher than that of CSA, while the oxidation degree is decreased. These results indicate that the level of sulfation is not the only factor controlling the oxidation percentage the process of free-radical depolymerization. Furthermore, as seen in Fig. 3, the β-linkage and higher degree of N-acetylated domains in CSA and OSCS lead to more oxidation, although these results do not suggest which is the primary factor.

Therefore, we prepared N-deacetylated heparan sulfate (HS-NH_2, Fig. S4). HS and HS-NH_2 were degraded with free radicals under the same conditions, and the oxidized oligomers accounted for 0.86 % and 0.17 %, respectively (Fig. S5). The oxidation percentage of HS, HS-NH_2, NS-heparosan, and heparin with different N-substituent groups and sulfation levels are far less than those of CSA and OSCS, suggesting that β-linkage rather than the N-substituent group is most important in the free-radical attack of the aldehyde end.

Dehydration initiated by radicals is preferred at an axial OH group, when the carbohydrates have a ring structure (Arts, Mombarg, vanBekkum, & Sheldon, 1997). The radical is shifted to C (1) with the loss of the axial OH group on C (1). All of the other radicals may rearrange through related processes (Parovuori, Hamunen, Forssell, Autio, & Poutanen, 1995), whereby ring scission occurs between C (5) and OC (1) when the C (6) radical undergoes rearrangement. The presence of ·OH radicals is the driving force of the reaction. The high reactivity of the radicals ensures a random attack of the polymer chain and degradation.

There is a certain similarity between free-radical degradation and ozone degradation (Fuchs & Schiller, 2014; Parovuori et al., 1995). The ROS can react with acetal functions (such as in carbohydrates) in a specific manner to yield esters. Ozonolysis has been reported to selectively depolymerize polysaccharides containing β-D-aldosidic linkages (Wang, Hollingsworth, & Kasper, 1998). The oxidation of aldosides is stereoselective and prefers the aglycone configuration, in which each oxygen atom has one of its electron lone-pair antiperiplanar to the alkylidene C–H bond. Therefore, glycosidic linkages in different configurations may exhibit differently reaction with ROS (Wardman & Candeias, 1996). GAGs with β-D-linked glycosides may be more susceptible to attack by ROS, resulting in an oxidized ring-opening structure (Fig. 4).

Furthermore, we discovered that there were microscale dehydration levels in the products of NS-heparosan and heparin, while these products were not detected in either CSA or OSCS. In addition, these dehydrated oligosaccharides in NS-heparosan and heparin also occurred aldonic acid terminals, as Fig. 5. (Zhang et al., 2008). The dehydrated oligosaccharides are detected in the products of NS-heparosan and heparin (Table S1) by the matching of exact molecular weight used bioinformatics software and mass spectrometry. However, due to the low abundance of dehydrated components, the multistage mass spectrometry analysis cannot detect their fragment ions and determine the specific location of the dehydration. This suggests that the dehydration may derive from the formation process of carbonyl-conjugated radicals or within the molecule (Arts et al., 1997; Parovuori et al., 1995).

4. Conclusions

Free-radical degradation of polysaccharides may result in non-specific and/or specific scission of carbohydrate chains, an action that is related to their structural units and special substituent groups. In this study, various GAGs were degraded utilizing a micro-reaction Fenton depolymerization process through copper ions catalysis, and then characterized using HILIC-FTMS. Comparing different types of GAG-derived oligosaccharides eluting on a HILIC column, the resolution characteristics of the HILIC column on acidic oligosaccharides were further investigated in detail. This examination demonstrated that the HILIC column could resolve the low degree of isomers, while this resolution is eliminated by high-sulfation. Meanwhile, the carboxyl modifications in the reducing end can significantly enhance the retention of oligosaccharides in a HILIC column, and their effect is much greater than that of sulfate modification. The profiling of different types of GAG-derived oligosaccharides also provides evidence for the free-radical depolymerization mechanism. The bulk of the fragments of GAGs generated by free-radical degradation maintains their basic structural units and sulfate substituents. While highly sulfated modification can resist the attack of free radicals on the backbone, the GAGs with low levels of sulfation are easily degraded into low molecular weight oligosaccharides. Furthermore, an abundance of oligomers modified with dehydration or oxidation in their reductive ends also appeared. The mixed oligosaccharides from CSA and OSCS can lead to oligosaccharides with aldonic acid terminal formation, while the products from heparin and NS-heparosan generally maintain their original structural units. These results indicate that, compared to the α-linked HexNY in GAGs, the β-linked structural units are easily oxidized into adaric acid. In addition, the results suggest that the low-sulfate domain is also advantageous for decreasing the degree of oxidation. Therefore, these findings will be useful in the identification of new GAGs as well as the preparation of low molecular weight derivatives or oligomers of GAGs.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2020.115847.

References

CID 53477715, https://pubchem.ncbi.nlm.nih.gov/compound/53477715.
CID 772, https://pubchem.ncbi.nlm.nih.gov/compound/772.
CID 4368136, https://pubchem.ncbi.nlm.nih.gov/compound/4368136.
Arts, S., Mombarg, E. J. M., vanBekkum, H., & Sheldon, R. A. (1997). Hydrogen peroxide and oxygen in catalytic oxidation of carbohydrates and related compounds. *Synthesis-Stuttgart,* (6), 597.
Bhaskar, U., Li, G. Y., Fu, L., Onishi, A., Suflita, M., Dordick, J. S., & Linhardt, R. J. (2015). Combinatorial one-pot chemoenzymatic synthesis of heparin. *Carbohydrate Polymers, 122*, 399–407.
Booth, F. W., & Thomason, D. B. (1991). Molecular and cellular adaptation of muscle in response to exercise - perspectives of various models. *Physiological Reviews, 71*(2), 541–585.
Chen, S. G., Li, G. Y., Wu, N. A., Guo, X., Liao, N. B., Ye, X. Q., & Chai, W. G. (2013). Sulfation pattern of the fucose branch is important for the anticoagulant and antithrombotic activities of fucosylated chondroitin sulfates. *Biochimica Et Biophysica Acta-General Subjects, 1830*(4), 3054–3066.
DeAngelis, P. L. (2012). Glycosaminoglycan polysaccharide biosynthesis and production: Today and tomorrow. *Applied Microbiology and Biotechnology, 94*(2), 295–305.
Fuchs, B., & Schiller, J. (2014). Glycosaminoglycan degradation by selected reactive oxygen species. *Antioxidants & Redox Signaling, 21*(7), 1044.
Furukawa, J., Okada, K., & Shinohara, Y. (2017). Glycomics of human embryonic stem cells and human induced pluripotent stem cells. *Glycoconjugate Journal, 34*(6), 807–815.
Galeotti, F., & Volpi, N. (2016). Oligosaccharide mapping of heparinase I-treated heparins by hydrophilic interaction liquid chromatography separation and online fluorescence detection and electrospray ionization-mass spectrometry characterization. *Journal of Chromatography A, 1445*, 68–79.
Gandhi, N. S., & Mancera, R. L. (2008). The structure of glycosaminoglycans and their interactions with proteins. *Chemical Biology & Drug Design, 72*(6), 455–482.
Green, D., Hull, R. D., Brant, R., & Pineo, G. F. (1992). Lower mortality in cancer-patients treated with low-molecular-Weight versus standard heparin. *Lancet, 339*(8807), 1476.
Jaitly, N., Mayampurath, A., Littlefield, K., Adkins, J. N., Anderson, G. A., & Smith, R. D. (2009). Decon2LS: An open-source software package for automated processing and visualization of high resolution mass spectrometry data. *BMC Bioinformatics, 10*, 15.
Jordan, K. M., Arden, N. K., Doherty, M., Bannwarth, B., Bijlsma, J. W. J., Dieppe, P., & Dougados, M. (2003). EULAR Recommendations 2003: An evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies including Therapeutic Trials (ESCISIT). *Annals of the Rheumatic Diseases, 62*(12), 1145–1155.
Kjellen, L., & Lindahl, U. (1991). Proteoglycans-structures and interactions. *Annual Review of Biochemistry, 60*, 443–475.
Leijdekkers, A. G. M., Sanders, M. G., Schols, H. A., & Gruppen, H. (2011). Characterizing plant cell wall derived oligosaccharides using hydrophilic interaction

chromatography with mass spectrometry detection. *Journal of Chromatography A, 1218*(51), 9227–9235.
Li, B. Z., Suwan, J., Martin, J. G., Zhang, F. M., Zhang, Z. Q., Hoppensteadt, D., & Linhardt, R. J. (2009). Oversulfated chondroitin sulfate interaction with heparin-binding proteins: New insights into adverse reactions from contaminated heparins. *Biochemical Pharmacology, 78*(3), 292–300.
Li, G. Y., Cai, C., Li, L. Y., Fu, L., Chang, Y. Q., Zhang, F. M., & Linhardt, R. J. (2014). Method to detect contaminants in heparin using radical depolymerization and liquid chromatography-mass spectrometry. *Analytical Chemistry, 86*(1), 326–330.
Li, L. Y., Ly, M., & Linhardt, R. J. (2012). Proteoglycan sequence. *Molecular BioSystems, 8*(6), 1613–1625.
Li, L. Y., Zhang, F. M., Zaia, J., & Linhardt, R. J. (2012). Top-down approach for the direct characterization of low molecular weight heparins using LC-FT-MS. *Analytical Chemistry, 84*(20), 8822–8829.
Ly, M., Leach, F. E., Laremore, T. N., Toida, T., Amster, I. J., & Linhardt, R. J. (2011). The proteoglycan bikunin has a defined sequence. *Nature Chemical Biology, 7*(11), 827–833.
Maxwell, E., Tan, Y., Tan, Y. X., Hu, H., Benson, G., Aizikov, K., & Zaia, J. (2012). GlycReSoft: A software package for automated recognition of glycans from LC/MS data. *PloS One, 7*(9), 11.
Merry, A. H., Neville, D. C. A., Royle, L., Matthews, B., Harvey, D. J., Dwek, R. A., & Rudd, P. M. (2002). Recovery of intact 2-aminobenzamide-labeled O-glycans released from glycoproteins by hydrazinolysis. *Analytical Biochemistry, 304*(1), 91–99.
Ofman, D., Slim, G. C., Watt, D. K., & Yorke, S. C. (1997). Free radical induced oxidative depolymerisation of chondroitin sulphate and dermatan sulphate. *Carbohydrate Polymers, 33*(1), 47–56.
Parovuori, P., Hamunen, A., Forssell, P., Autio, K., & Poutanen, K. (1995). Oxidation of potato starch by HYDROGEN-PEROXIDE. *Starch-Starke, 47*(1), 19–23.
Quader, M. A., Stump, L. S., & Sumpio, B. E. (1998). Low molecular weight heparins: Current use and indications. *Journal of the American College of Surgeons, 187*(6), 641–658.
Richy, F., Bruyere, O., Ethgen, O., Cucherat, M., Henrotin, Y., & Reginster, J. Y. (2003). Structural and symptomatic efficacy of glucosamine and chondroitin in knee osteoarthritis - A comprehensive meta-analysis. *Archives of Internal Medicine, 163*(13), 1514–1522.
Saad, O. M., Ebel, H., Uchimura, K., Rosen, S. D., Bertozzi, C. R., & Leary, J. A. (2005). Compositional profiling of heparin/heparan sulfate using mass spectrometry: Assay for specificity of a novel extracellular human endosulfatase. *Glycobiology, 15*(8), 818–826.
Sanderson, P., Stickney, M., Leach, F. E., Xia, Q. W., Yu, Y. L., Zhang, F. M., & Amster, I. J. (2018). Heparin/heparan sulfate analysis by covalently modified reverse polarity capillary zone electrophoresis-mass spectrometry. *Journal of Chromatography A, 1545*, 75–83.
Shi, X. F., & Zaia, J. (2009). Organ-specific heparan sulfate structural phenotypes. *The Journal of Biological Chemistry, 284*(18), 11806–11814.
Silva, J. C., Carvalho, M. S., Han, X. R., Xia, K., Mikael, P. E., Cabral, J. M. S., ... Linhardt, R. J. (2019). Compositional and structural analysis of glycosaminoglycans in cell-derived extracellular matrices. *Glycoconjugate Journal, 36*(2), 141–154.
Staples, G. O., Naimy, H., Yin, H. F., Kileen, K., Kraiczek, K., Costello, C. E., & Zaia, J. (2010). Improved hydrophilic interaction chromatography LC/MS of heparinoids using a chip with postcolumn makeup flow. *Analytical Chemistry, 82*(2), 516–522.
Uebelhart, D. (2008). Clinical review of chondroitin sulfate in osteoarthritis. *Osteoarthritis and Cartilage, 16*, S19–S21.
van Schaick, G., Pirok, B. W. J., Haselberg, R., Somsen, G. W., & Gargano, A. F. G. (2019). Computer-aided gradient optimization of hydrophilic interaction liquid chromatographic separations of intact proteins and protein glycoforms. *Journal of Chromatography A, 1598*, 67–76.
Vismara, E., Pierini, M., Guglieri, S., Liverani, L., Mascellani, G., & Torri, G. (2007). Structural modification induced in heparin by a Fenton-type depolymerization process. *Seminars in Thrombosis and Hemostasis, 33*(5), 466–477.
Vismara, E., Pierini, M., Mascellani, G., Liverani, L., Lima, M., Guerrini, M., & Torri, G. (2010). Low-molecular-weight heparin from Cu2+ and Fe2+ Fenton type depolymerisation processes. *Thrombosis and Haemostasis, 103*(3), 613–622.
Wang, Y., Hollingsworth, R. I., & Kasper, D. L. (1998). Ozonolysis for selectively depolymerizing polysaccharides containing beta-D-aldosidic linkages. *Proceedings of the National Academy of Sciences of the United States of America, 95*(12), 6584–6589.
Wardman, P., & Candeias, L. P. (1996). Fenton chemistry: An introduction. *Radiation Research, 145*(5), 523–531.
Wing, D. R., Garner, B., Hunnam, V., Reinkensmeier, G., Andersson, U., Harvey, D. J., ... Butters, T. D. (2001). High-performance liquid chromatography analysis of ganglioside carbohydrates at the picomole level after ceramide glycanase digestion and fluorescent labeling with 2-aminobenzamide. *Analytical Biochemistry, 298*(2), 207–217.
Wuhrer, M., Koeleman, C. A. M., Deelder, A. M., & Hokke, C. N. (2004). Normal-phase nanoscale liquid chromatography - Mass spectrometry of underivatized oligosaccharides at low-femtomole sensitivity. *Analytical Chemistry, 76*(3), 833–838.
Yip, G. W., Smollich, M., & Gotte, M. (2006). Therapeutic value of glycosaminoglycans in cancer. *Molecular Cancer Therapeutics, 5*(9), 2139–2148.
Zhang, Z. Q., McCallum, S. A., Xie, J., Nieto, L., Corzana, F., Jimenez-Barbero, J., & Linhardtt, R. J. (2008). Solution structures of chemoenzymatically synthesized heparin and its precursors. *Journal of the American Chemical Society, 130*(39), 12998–13007.
Zhao, X., Yang, B., Li, L. Y., Zhang, F. M., & Linhardt, R. J. (2013). On-line separation and characterization of hyaluronan oligosaccharides derived from radical depolymerization. *Carbohydrate Polymers, 96*(2), 503–509.
Zhi, Z. J., Li, J. H., Chen, J. L., Li, S., Cheng, H., Liu, D. H., & Chen, S. G. (2019). Preparation of low molecular weight heparin using an ultrasound-assisted Fenton-system. *Ultrasonics Sonochemistry, 52*, 184–192.