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Molecular Mass Characterization of Glycosaminoglycans with Different Degrees of Sulfation in Bioengineered Heparin Process by Size Exclusion Chromatography

Zhenyu Wang[1,5], **Fuming Zhang**[3,5], **Jonathan S. Dordick**[1,3,4,5], and **Robert J. Linhardt**[1,2,3,4,5,*]

[1]Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12180, USA

[2]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, New York 12180, USA

[3]Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York 12180, USA

[4]Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, New York 12180, USA

[5]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, USA

Abstract

Different degrees of glycosaminoglycan sulfation result in their different charge densities. The charge density differences impact their migration behavior in polyacrylamide gel electrophoresis and size exclusion chromatography, two of the most common methods for determining relative molecular masses of polysaccharides. In this study, we investigated the feasibility of using commercially available heparin oligosaccharides as calibrants for measuring the relative molecular masses of intermediates in a bioengineered heparin process that have different levels of sulfation. A size exclusion chromatography method was established that eliminates this charge density effect and allows the determination of relative molecular mass using a single calibration curve with heparin oligosaccharides calibrants. This is accomplished by overcoming the electrostatic interaction between the glycosaminoglycans and size exclusion chromatography stationary phase using high ionic strength mobile phase.

Keywords

Bioengineered heparin; charge density; electrophoresis; electrostatic interaction; glycosaminoglycan; heparin; heparosan; molecular mass; polysaccharide; size exclusion chromatography

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*Address correspondence to this author at the Rensselaer Polytechnic Institute, Biotech Center 4005, 110 8th Street, Troy, NY 12180-3590, USA; Tel: 518-276-3404; Fax: 518-276-3405; linhar@rpi.edu.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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INTRODUCTION

Glycosaminoglycans (GAGs) are long unbranched polysaccharides consisting of a repeating disaccharide unit. The repeating unit consists of a hexose or a hexuronic acid, linked to a hexosamine. In most cases, the disaccharide unit of a GAG is sulfated. GAGs were classified based on their disaccharide composition, linkage type and pattern of sulfate group substitution. Examples of GAGs include: (1) hyaluronan, (2) chondroitin, chondroitin sulfate and dermatan sulfate, and (3) heparosan, heparan sulfate and heparin (Table **1**). All of these GAGs, with the exception of hyaluronan, chondroitin and heparosan, contain sulfate groups. The degree of sulfation for each GAG class usually varies according to the source of the raw material where GAGs are extracted and the method used to prepare the GAGs [1].

Average relative molecular mass is an important parameter for GAGs with therapeutic applications. For example, the GAG heparin produces its anticoagulant effect by inhibiting activated factor X (factor Xa) and thrombin through the binding and activation of antithrombin (AT). Antithrombin is a member of the serpin family of serine proteinase inhibitors with primary physiological function of inhibiting and regulating several serine proteinases in the blood coagulation pathway [2]. Heparin binds to AT through a high-affinity pentasaccharide sequence, which is present on about a third of heparin molecules. For inhibition of thrombin, heparin must bridge between thrombin and AT, whereas bridging is not required for inhibiting Xa. Chains of heparin with fewer than 18 saccharide units lack the requisite chain length to bridge between thrombin and AT and, therefore, are unable to inhibit thrombin. In contrast, very small heparin chains, or even heparin oligosaccharides, containing a specific AT-binding pentasaccharide sequence can catalyze the AT-mediated inhibition of factor Xa. By inhibiting thrombin, heparin not only prevents fibrin formation but also inhibits thrombin-induced activation of platelets and of factors V and VIII [3]. The relative molecular mass of another GAG with wide medical and cosmetic applications, hyaluronan (HA), also is related to its physiochemical and biological properties. High relative molecular mass HA has unique rheological properties required for mucoadherence, and its anti-inflammatory activity, whereas low relative molecular mass HA is a potent signaling molecule [4]. Thus, determining the relative molecular mass properties of GAGs can be important in characterizing their activities.

A number of techniques are available for characterizing the relative molecular mass of polysaccharides. These techniques include electrophoresis, size exclusion chromatography (SEC), viscosimetry, mass spectrometry and light scattering. Mass spectrometry and static light scattering afford an absolute measure of relative molecular mass. Thus, these methods do not require standards or calibrants to calculate GAG relative molecular mass. Unfortunately, current mass spectrometric techniques are often limited by the size of the molecule and cannot detect high relative molecular mass GAGs [5]. Moreover, static light scattering is only effective in measuring the weight average relative molecular mass (M_w) of large molecules. The coupling of multiangle static light scattering to SEC can afford additional relative molecular mass properties such as number average relative molecular mass (M_n) and polydispersity (PD) [6]. However, multiangle light scattering systems are expensive and are usually only found in laboratories specialized in polymer characterization. The most accessible techniques for relative molecular mass measurement to the majority of biochemical laboratories are gel electrophoresis and SEC. The migration rate of a molecule in gel electrophoresis is determined by its charge, shape and size when the electric field and gel matrix are fixed [7]. The separation in SEC depends on the different abilities of the various sample molecules to enter the pores of the stationary phase: small molecules can access more pores of the stationary phase than large molecules and, thus, move slower through the chromatographic bed. Therefore, in SEC, samples are eluted in the order of decreasing molecular size, and the elution time can be used to assess the sample relative

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molecular mass when calibrants of know relative molecular mass are available [8]. However, in SEC, factors other than molecular size such as electrostatic interaction of analyte with the stationary phase can also impact the retention time. The extent of these effects and ways to overcome them have been discussed in a number of publications [9–11].

Heparin is currently produced from a porcine intestinal extract. Its animal sourced production is associated with several drawbacks including issues in quality control and source material availability, which were brought to international attention during the heparin contamination crisis of 2008 [1]. Our laboratory proposed a new approach to produce bioengineered heparin from a non-animal source to overcome these drawbacks. The preparation of bioengineered heparin starts with *E. coli* K5 fermentation to produce the heparosan precursor, followed by a series of chemo-enzymatic steps to alter the stereochemistry and add sulfate groups into the heparosan backbone to afford bioengineered anticoagulant heparin [12–14]. The relative molecular mass properties of the bioengineered heparin need to closely match that of the porcine heparin to make the bioengineered heparin identical to the United States Pharmacopoeia (USP) porcine heparin. Therefore, the control of relative molecular mass properties of polysaccharide throughout the bioengineered heparin process is critical. The polysaccharides in the bioengineered heparin process include: heparosan; *N*-sulfo ($N\text{-}SO_3^-$) heparosan; undersulfated heparin; heparin; and anticoagulant heparin. The degree of sulfation increases with each process step. Relative molecular mass standards corresponding to each different polysaccharide are typically required to characterize the relative molecular mass properties of bioengineered heparin and all process intermediates using gel electrophoresis and SEC. However, there are no commercially available relative molecular mass standards for heparosan and each process intermediate. This study investigated the feasibility of using the commercially available heparin oligosaccharides as calibrants for measuring the relative molecular mass of heparosan and bioengineered heparin process intermediates by gel electrophoresis and SEC.

MATERIALS AND METHODS

Materials

Electrophoresis-grade acrylamide, N,N'-methylenebisacrylamide, sucrose, glycine, ammonium persulfate (APS), N,N,N',N'-tetramethylenediamine (TEMED), were from Bio-Rad (Hercules, CA, USA). Phenol red, and Alcian blue were from Fisher (Pittsburgh, PA, USA). SEC column TSK-GEL G3000PWxl was from Tosoh Bioscience (King of Prussia, PA). $NaNO_3$ was from Sigma–Aldrich (St. Louis, MO). Heparin oligosaccharides were from Iduron (Manchester, UK). Heparosan oligosaccharides were prepared in our laboratory with continuous-elution preparative PAGE [5].

Isocratic Large-Slab Polyacrylamide Electrophoresis—The resolving 16 cm × 16 cm × 0.75 cm slab gels were cast from 25 mL of a 10% m/v total acrylamide (10% T) monomer solution containing 9.66% (m/v) acrylamide and 0.33% (m/v) N,N'-methylene-bis-acrylamide, 125 μL 10% m/v APS and 12.5 μL TEMED. The stacking gel was cast from 10 mL of 5% T solution with 50 μL 10% m/v APS and 10 μL TEMED. Oligosaccharide samples (~5 ug each) and polysaccharide sample (~100 ug) were mixed with phenol red and sucrose respectively before loaded onto the gel. The gel was subjected to electrophoresis using a Protean II Xi Cell (Bio-Rad, Hercules, CA, USA) apparatus at 400 V for 1.25 h, and its inner core was cooled with cold tap water. The upper chamber buffer contains 1 M glycerol and 0.2 M Tris, and the lower chamber buffer contains 0.1 M Tris-borate and 0.001 M EDTA. After staining overnight in a solution of 0.5% (m/v) Alcian blue in 2% m/v acetic acid, the gels were destained in water [5].

SEC—SEC was performed using TSK-GEL G3000PWxl size exclusion column with a sample injection volume of 20 μL and a flow rate of 0.6 ml/min on an apparatus composed of a Shimadzu LC-10Ai pump, a Shimadzu CBM-20A controller and a Shimadzu RID-10A refractive index detector. Oligosaccharide samples were reconstituted to a concentration of 1 mg/ml before injection. The mobile phase consisted of 0.1 M or 2.0 M $NaNO_3$. The column was maintained at 40°C with an Eppendorf column heater during the chromatography. The SEC chromatograms were recorded with the LCsolution version 1.25 software and analyzed with its "GPC Postrun" function.

RESULTS AND DISCUSSION

Gel Electrophoresis

The charge density in heparosan, process intermediates, and heparin differ due to the differences in their content of sulfate groups. Heparin moves faster than heparosan with similar relative molecular mass in electrophoresis due to its higher charge density. For example, a heparin oligosaccharide with relative molecular mass of 5.8 kDa migrated at similar speed with a heparosan oligosaccharide with much lower relative molecular mass of 3.8 kDa in a 10% m/v isocratic large-slab polyacrylamide gel (Fig. **1**). Thus, gel electrophoresis for relative molecular mass measurement of heparosan, process intermediates and heparin using available heparin oligosaccharide standards as calibrants is not reliable since these are substituted with different number of sulfate groups.

SEC—Even in SEC with linear polysaccharides of identical chain length and having the same linkage configuration and position, the number and distribution of charged sulfate groups can have a profound impact on movement through a porous gel. An increased number of sulfate groups can impact molecular shape, molecular size of a given oligosaccharide or polysaccharide chain by promoting undesired electrostatic interactions between the solute and stationary support. A universal calibration method for the SEC measurement of the relative molecular mass properties of heparosan, process intermediates and heparin was further investigated.

We hypothesized that the relative molecular mass properties of heparosan and process intermediates could be measured by SEC using a plot of log (MW) *versus* retention time of commercial heparin oligosaccharide calibrants, since heparosan and process intermediates have a similar molecular shape and backbone structure to that of heparin. Heparin oligosaccharides of different relative molecular masses (1.61 kDa, 2.69 kDa, 4.30 kDa and 5.38 kDa) were injected onto TSK-GEL G3000PWxl column with 0.1 M $NaNO_3$ as mobile phase and the log (M_r) was plotted as a function of retention time. Sodium nitrate is a stable, non-reactive, water soluble salt that is compatible with carbohydrates and is not corrosive to the stainless steel present in most commercial HPLC systems. Heparosan oligosaccharides, prepared in our laboratory and having relative molecular masses of 1.14 kDa, 1.90 kDa, 3.03 kDa and 3.41 kDa, were also injected onto the TSK-GEL G3000PWxl column in 0.1 M $NaNO_3$ mobile phase, and their log (M_r) was plotted as a function of retention time. The linear plots of log(M_r) *versus* time for heparin and heparosan calibrants diverged (Fig. **2**), indicating that under these analytical conditions a single standard curve using heparin calibrants could not be used to accurately determine the relative molecular mass properties of heparosan nor would such a standard curve be useful for the analysis of undersulfated process intermediates. Under these conditions, heparin elutes from SEC column earlier than heparosan of comparable molecular mass. We suggest that this is primarily due to the highly negative charge that heparin carries. Heparin's negative charge can result in electrostatic repulsion with the stationary phase, hydroxylated polymethacrylate. Electrostatic repulsion impedes heparin's ability to enter the pore structure of the stationary phase, resulting in a

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shorter flow path and reduced retention time [15]. Heparosan and its sulfated derivatives have different charge densities, thus the effect of their electrostatic repulsion would undoubtedly result in retention times that are different even for molecules of identical relative molecular mass, preventing the use of a single standard curve to determine the relative molecular mass of all these analytes.

The calibration plots of heparosan standards (having the lowest negative charge in the bioengineered heparin process) and heparin standards (having the highest negative charge in the bioengineered heparin process) need to merge into a single linear calibration plot to accurately measure the relative molecular mass properties of heparosan, the process intermediates and heparin using a single set of commercially available heparin oligosaccharide calibrants. We hypothesized that the electrostatic interaction between anionic polysaccharide analyte and stationary phase could be suppressed by using a mobile phase of high ionic strength. A new mobile phase, 2 M $NaNO_3$, was selected for this study. Using a high ionic strength mobile phase, the retention time of both heparin and heparosan were found to increase. For example, a heparin oligosaccharide having a relative molecular mass of 2.69 kDa eluted from the column at 12.93 min using 0.1 M $NaNO_3$ mobile phase, and at 13.97 min in using 2 M $NaNO_3$ mobile phase (Fig. **3**). Similarly, a heprosan oligosaccharide having a relative molecular mass of 1.90 kDa eluted from the column at 13.44 min using 0.1 M $NaNO_3$ and at 14.20 min using 2 M $NaNO_3$ (data not shown). In both cases, the increased ionic strength of mobile phase suppressed the electrostatic repulsion between the stationary phase and the negative charge from heparin's carboxylate and sulfate groups and heparosan's carboxylate groups. At 2 M, the $NaNO_3$ mobile phase, merged both log(MW) *versus* retention time plots, for heparin and heparosan oligosaccharides, into a single linear curve (Fig. **4**). These results suggest that the electrostatic interactions of heparosan and heparin with the stationary phase have been eliminated allowing a single standard curve to be used to calculate the relative molecular mass properties of both polysaccharides as well as the process intermediates. It is also possible to overcome the electrostatic interaction by neutralizing the stationary phase surface charge with low pH mobile phase, although low pH may pose the risk of changing the analytes' molecular mass by depolymerization and/or desulfation [16]. Hydrodynamic volume difference between heparosan, heparin and process intermediates, may exert a second order effect on the SEC retention time and may require additional studies to define their impact. A more accurate calibration curve may use the plot of $\log([\eta]M)$ *versus* retention time, where $[\eta]$ denotes the intrinsic viscosity and M is the weight average relative molecular mass. The term $[\eta]M$ is proportional to the solute's hydrodynamic volume, and Benoit *et al.* [17] demonstrated that a number of polymers with different configurations (e.g., linear, comb-branched, star-branched) all conformed to a single plot of $\log([\eta]M)$ *versus* the elution volume. However, intrinsic viscosity measurement usually requires large quantities of sample, which is very costly for heparin and heparosan oligosaccharides.

CONCLUSIONS

A simple SEC method was established using the commercially available heparin oligosaccharides as calibrants to measure the relative molecular mass of GAGs with different degree of sulfation involved in the bioengineered heparin process. The SEC method eliminated the charge density effect using high ionic strength mobile phase and allowed a single calibration curve with heparin oligosaccharides to measure the GAGs in the bioengineered heparin process, without the need for complicated instrumentation and only requires microgram scale amount of sample.

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Acknowledgments

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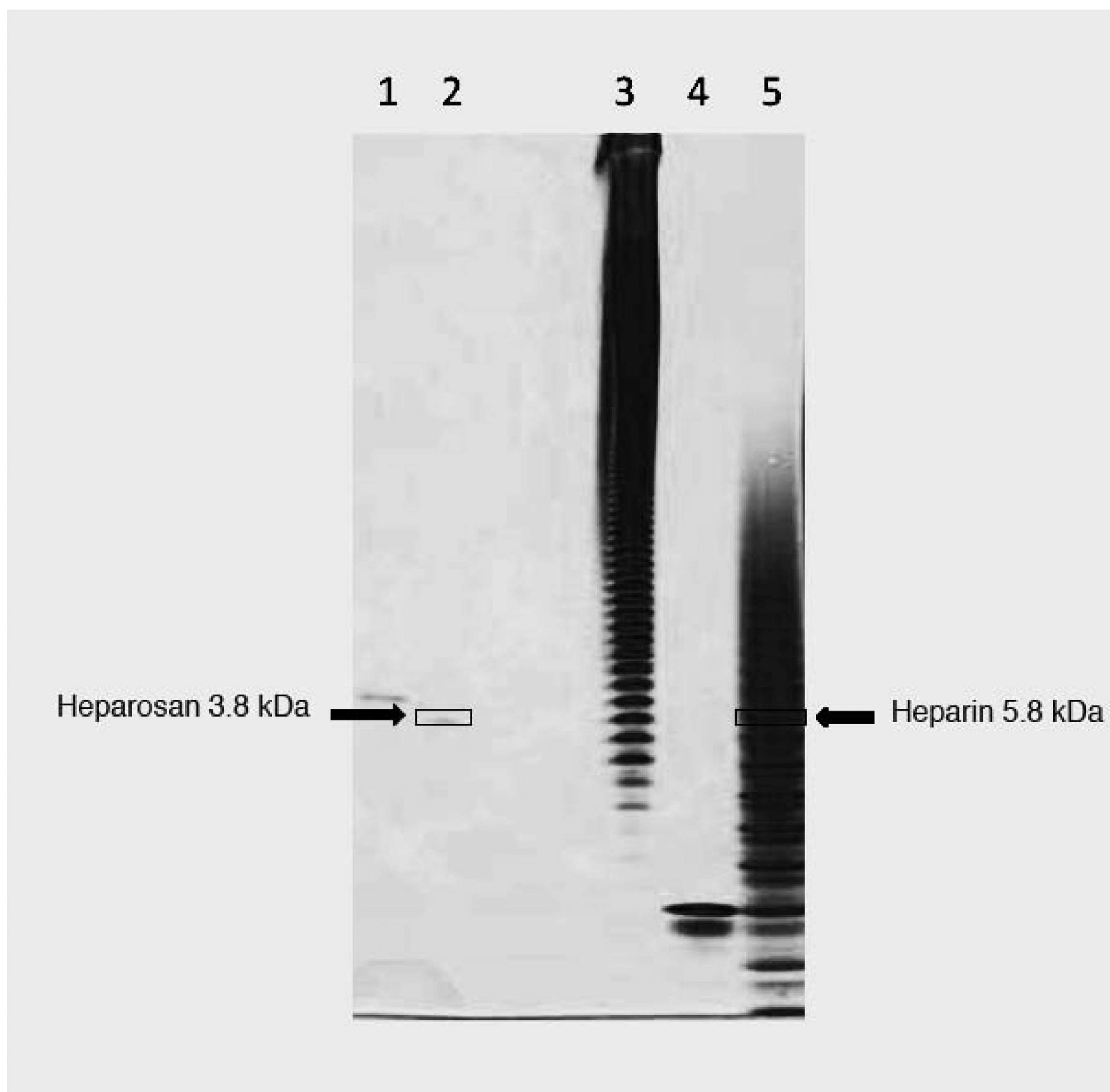

Fig. (1).

Electrophoresis of heparosan and heparin samples on a 10% m/v large-slab polyacrylamide gel. Lane 1: heparosan oligosaccharide with relative molecular mass of 4.2 kDa, lane 2: heparosan oligosaccharide with relative molecular mass of 3.8 kDa, lane 3: heparosan polysaccharide produced from *E. coli* K5, lane 4: heparin oligosaccharide (the upper and most intense band) with relative molecular mass of 2.3 kDa, lane 5: bovine lung heparin ladder. The gel was run at 400 V for 1.25 h. Note that heparosan of 3.8 kDa and heparin of 5.8 kDa migrated at similar rate.

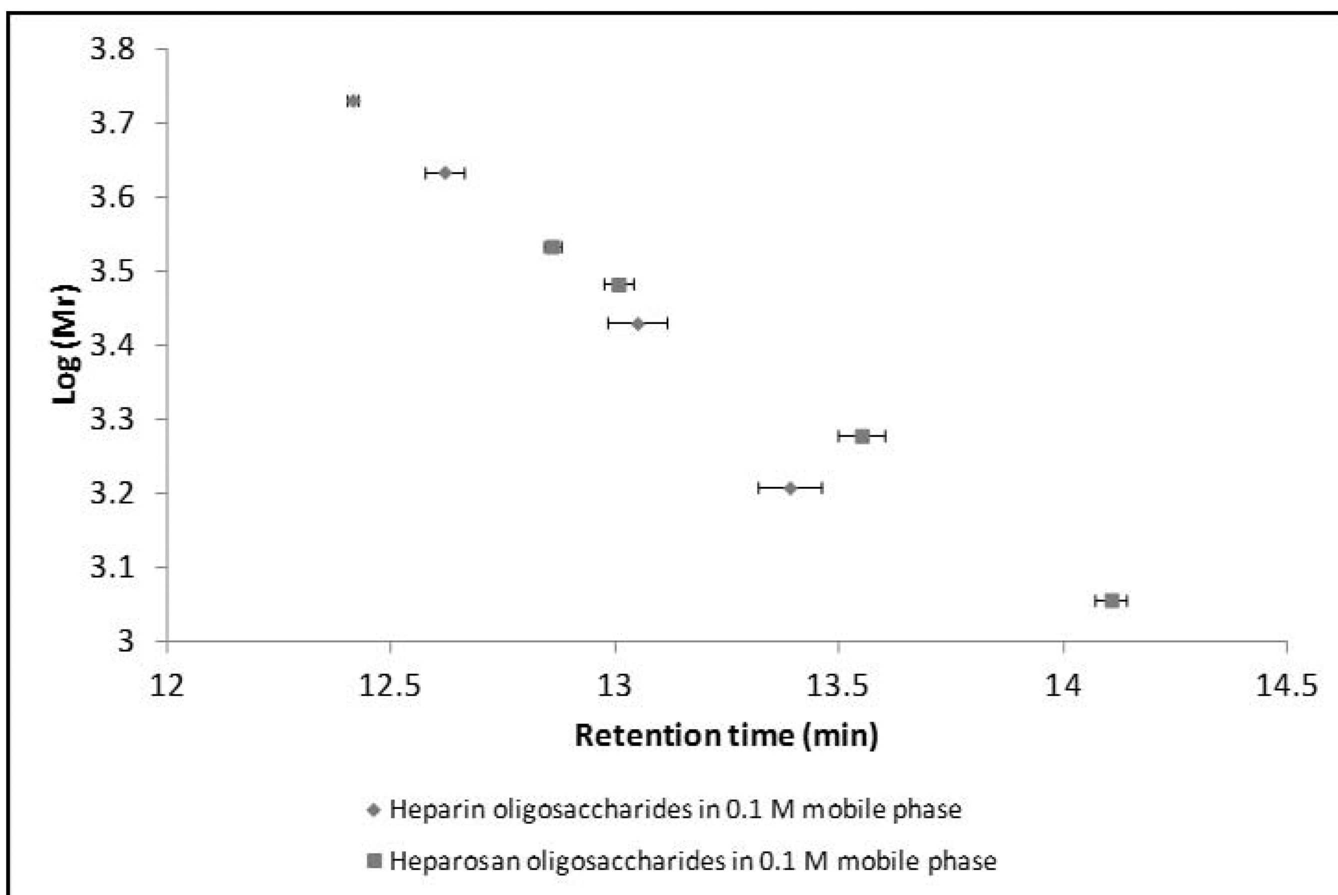

Fig. (2).

Plots of log(M_r) versus retention time for heparin and heparosan standard oligosaccharides, respectively, from TSK-GEL G3000PWxl column in 0.1 M $NaNO_3$ mobile phase. The horizontal error bars represent standard deviation from the mean retention time of duplicate runs.

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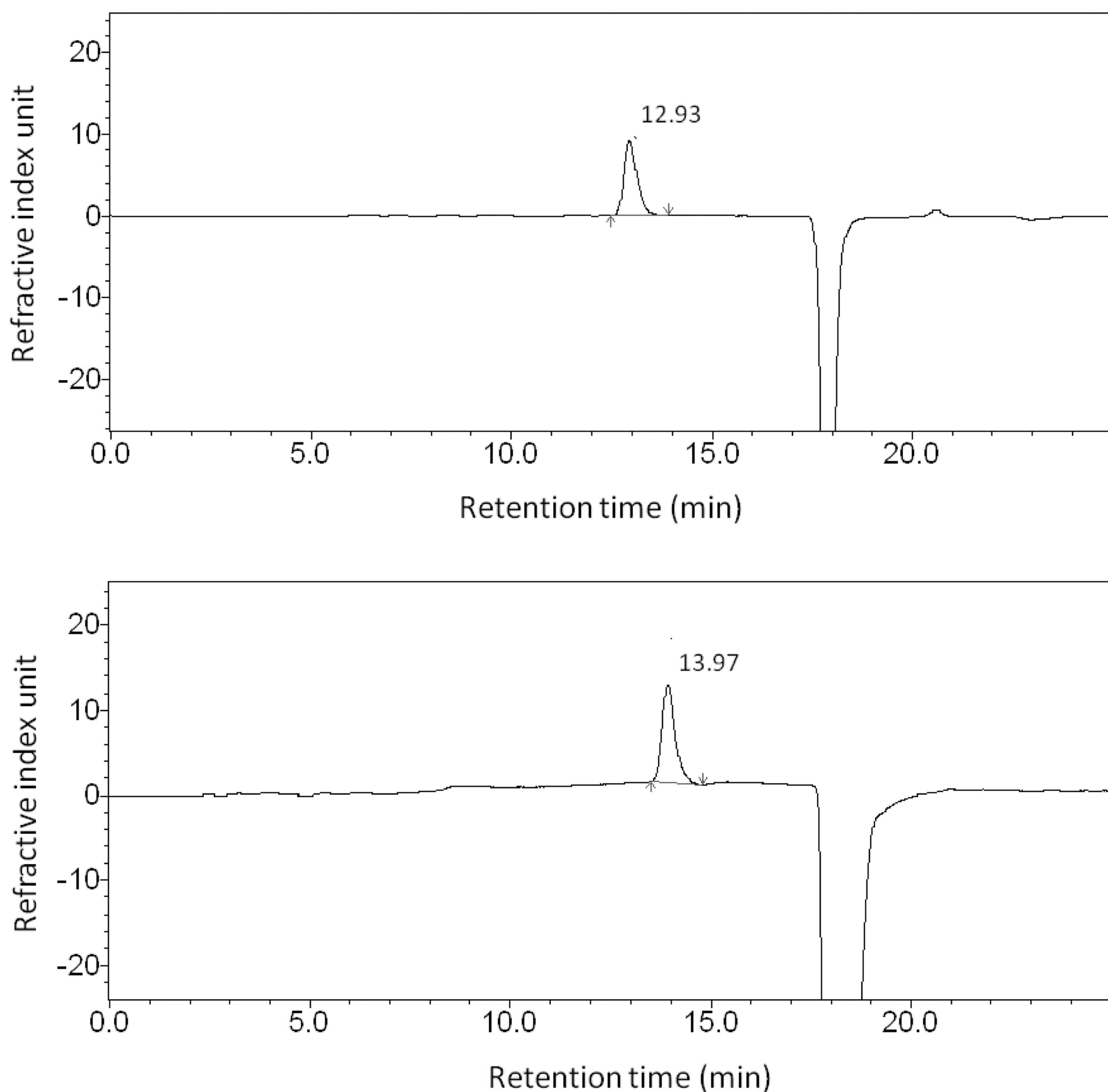

Fig. (3).
a) SEC chromatogram of heparin oligosaccharide of relative molecular mass 2.69 kDa in 0.1 M $NaNO_3$ mobile phase. b) SEC chromatogram of heparin oligosaccharide of relative molecular mass 2.69 kDa in 2 M $NaNO_3$ mobile phase.

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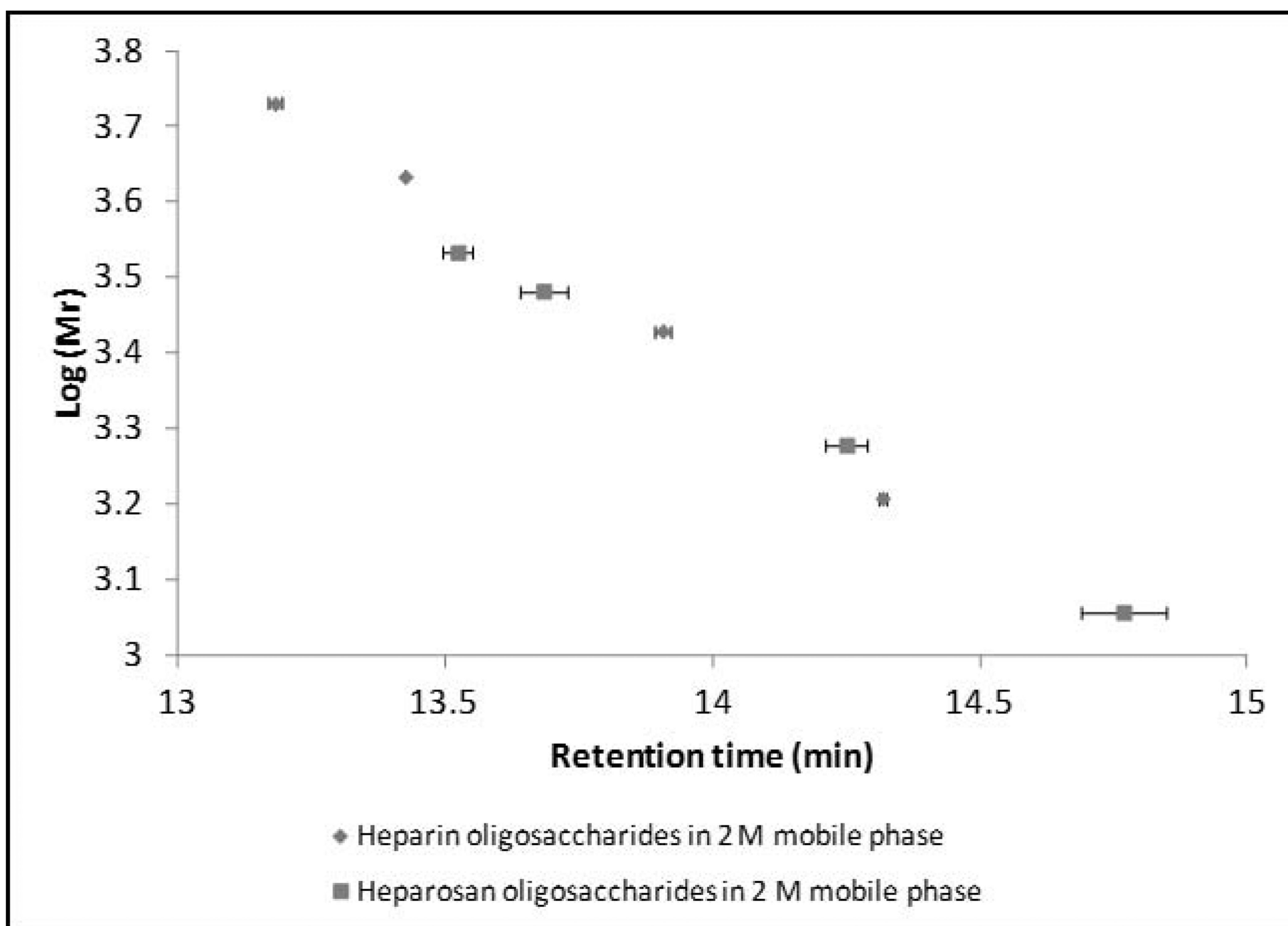

Fig. (4).

Plots of log(M_r) versus retention time for heparin and heparosan standard oligosaccharides, respectively, from TSK-GEL G3000PWxl column in 2 M $NaNO_3$ mobile phase. The horizontal error bars represent standard deviation from the retention time of duplicate runs.

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Table 1

Major Repeating Structures of Uronic Acid Containing Glycosaminoglycans[a]

GAG	Linkage	Sugar 1	Sulfates	Linkage	Sugar 2	Sulfates	Level of Sulfation
hyaluronan	(1→3)	GlcNAc	none	(1→4)	GlcA	none	0
chondroitin	(1→3)	GalNAc	none	(1→4)	GlcA	none	0
chondroitin sulfate	(1→3)	GalNAc	4S or 6S	(1→4)	GlcA	none	1
dermatan sulfate	(1→3)	GalNAc	4S	(1→4)	IdoA or GlcA	none	1
heparaosan	(1→4)	GlcNAc	none	(1→4)	GlcA	none	0
							1
heparan sulfate	(1→4)	GlcNAc or NS	none or 6S or 3S	(1→4)	GlcA	none or 2S	1
heparin	(1→4)	GlcNS or GlcNAc	6S ± 3S	(1→4)	IdoA or GlcA	2S	3
***N*-sulfo heparosan**	(1→4)	GlcNS	none	(1→4)	GlcA	none	1
undersulfated heparin	(1→4)	GlcNS or GlcNAc	none or 6S or 3S	(1→4)	GlcA or IdoA	none or 2S	2

[a]Only the major form of each polysaccharide is shown. Some structural variability is indicated when a second sugar type or a second sulfate group position is indicated after the word "or". The level of sulfation is the average number of sulfates in the disaccharide unit the major form of each GAG. The 3S groups are only rarely found in heparin and heparin sulfate and are required for their binding to anthrombin. The *N*-sulfoheparoan and undersulated heparin are intermediates of the bioengineered heparin process. Abbreviations are: GlcNAc, *N*-acetyl-α-D-glucosamine; GalNAc, *N*-acetyl–β-D-galactosamine; GlcNS, *N*-sulfo–α-D-glucosamine; GlcA, β-D-glucuronic acid; α-L-IdoA iduronic acid; and S, sulfo.