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RESEARCH PAPER

Capillary electrophoresis for total glycosaminoglycan analysis

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Abstract A capillary zone electrophoresis–laser-induced fluorescence detection (CZE-LIF) method was developed for the simultaneous analysis of disaccharides derived from heparan sulfate, chondroitin sulfate/dermatan sulfate, hyaluronan, and keratan sulfate. Glycosaminoglycans (GAGs) were first depolymerized with the mixture of GAG lyases (heparinase I, II, III and chondroitinase ABC and chondroitinase AC II) and GAG endohydrolase (keratinase II) and the resulting disaccharides were derivatized by reductive amination with 2-aminoacridone. Nineteen fluorescently labeled disaccharides were separated using 50 mM phosphate buffer (pH 3.3) under reversed polarity at 25 kV. Using these conditions, all the disaccharides examined were baseline separated in less then 25 min. This CZE-LIF method gave good reproducibility for both migration time (≤1.03 % for intraday and ≤4.4 % for interday) and the peak area values (≤5.6 % for intra- and ≤8.69 % for interday). This CZE-LIF method was used for profiling and quantification of GAG derivative disaccharides in bovine cornea. The results show that the current CZE-LIF method offers fast, simple, sensitive, reproducible determination of disaccharides derived from total GAGs in a single run.

Keywords Capillary electrophoresis · Biopolymers · Enzymes

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Introduction

Glycosaminoglycans (GAGs) are linear, highly charged, anionic polysaccharides consisting of repeating disaccharides units and can be divided into four classes—heparan sulfate (HS)/heparin (HP), chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (HA). These classes differ in the structure of their repeating disaccharides and also in their biological functions [1]. They are widely represented on cell surfaces, inside cells, and in the extracellular matrix. GAGs, with the exception of hyaluronan, are biosynthesized as protein/GAG glycoconjugates known as proteoglycans. The biological functions of proteoglycans are principally determined by the structure of their GAG chains [2, 3].

GAGs interact with hundreds of plasma proteins, including growth factors, cytokines, chemokines, proteases, protease inhibitors, coagulant and anticoagulant proteins, complement proteins, lipoproteins, and lipolytic enzymes. These interactions are critical in cell adhesion, proliferation, motility and differentiation, viral and bacterial infection, cancer, and inflammation [4, 5]. Therefore, considerable attention has been focused on qualitative and quantitative analysis of GAGs to understand their biological importance.

Analysis of intact GAGs are challenging because they have a highly negative charge, a polydisperse and microheterogeneous structure, and a high molecular weight [6]. Therefore, instead of analyzing intact GAGs, analysis is usually performed after chemically or enzymatically depolymerizing GAGs to oligosaccharides or disaccharides. Knowing the structure and amount of each disaccharide or oligosaccharide unit can be useful for understanding the structure–activity relationship of GAGs [7, 8].

GAG-degrading enzymes are divided into two classes—GAG lyases and GAG hydrolases. There are several common GAG-degrading endolytic lyases: (1) heparinases (I, II, and III), acting on HS and HP; (2) the chondroitinases (ABC and ACII) acting primarily on CS and DS; and (3) bacterial hyaluronidases acting primarily on HA. It is noteworthy that chondroitinases can act on linkages within HA and that bacterial hyaluronidases can act on linkages within CS [9]. GAG-degrading lyases eliminatively cleave glycosidic linkages between hexosamine and hexuronic acid residues, leaving a deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid (ΔUA) residue at the nonreducing end of the newly formed product [9, 10].

GAG hydrolase enzymes act by hydrolytic mechanism to produce saturated residues at the products nonreducing ends. Keratanase and keratinase II are the GAG endohydrolases that depolomerize KS. Keratanase cleaves the internal β1–4 galactosidic linkages in KS and generally affords oligosaccharides. In contrast, keratanase II cleaves β1–3 glucosaminidic linkages to galactose, as well as those in KS to galactose-6-sulfate, releasing monosulfated or disulfated disaccharides from KS [11].

Following GAG depolymerization, the separation of disaccharide product often relies on strong anion exchange, reversed-phase, reversed-phase ion-pair, normal phase, high-performance liquid chromatography (HPLC) and hydrophilic interaction chromatography, capillary electrophoresis (CE), and fluorophore-assisted carbohydrate electrophoresis [12–42]. Mass spectrometry, ultraviolet and fluorescence detection methods are then used for analyte detection and quantification. Enzymatic depolymerization of all GAGs, with the exception KS, results in products containing a ΔUA residue at their nonreducing end, making these detectable in the ultraviolet (UV) at 232 nm (ε_M~3,000–5,000). However, the analysis of the low quantities of GAGs, present in a small number of cells, requires higher detection sensitivity. Therefore, precolumn or postcolumn derivatization is frequently used in disaccharide analysis [21–41]. These derivatization methods not only enhance detection sensitivity but can also improve disaccharide separations [43]. A number of fluorophores, including 2-cyanoacetamide [26], 2-aminobenzoic acid [27, 37], 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene hydrazide derivatives [28], 2-aminopyridine [29], 2-aminoacridone (AMAC) [22–25, 30–36], 7-aminonaphthalene-1,3-disulfonic acid [39], and 8-aminonaphthalene-1,3,6-trisulfonic acid [41] are commonly used for labeling GAG-derived disaccharides.

CE is a powerful technique that has some advantages over other chromatographic approaches, such as high separation efficiency, rapid analysis, relatively simple instrumentation, and low sample consumption. CE, combined with laser-induced fluorescence (LIF) detector, becomes a very sensitive method for the disaccharide analysis. LIF detection of AMAC-derivatized GAG disaccharides is up to 100-fold more sensitive than UV detection of the nonderivatized GAG disaccharides at 232 nm [32]. Disaccharides derived from CS/DS, HS/HP, and HA are typically analyzed by CE-UV or CE-LIF in a separate CE experiments [30–42]. This requires multiple, repetitive, and tedious sample preparation steps, including GAG extraction from biological sample, GAG depolymerization, disaccharide labeling, and disaccharide analysis. These multiple analytical procedures results in lost time, lost sample, reduced sensitivity, and higher costs.

There is a recent report that uses CZE-LIF for the simultaneous analysis of 17 AMAC-labeled CS/DS, HA, and HS disaccharides [30]. In this report, the total analysis time is more than 60 min and also disaccharides derived from KS were not determined. The only method, which has been used for the simultaneous analysis of KS, HS/HP, and CS/DS disaccharides in a single run, relies on liquid chromatography tandem mass spectrometry (LC-MS-MS), and this method only resolved a seven-component mixture of NS_{HS}, $0S_{HS}$, $6S_{HS}$, $4S_{CS}$, $6S_{CS}$, $2S_{KS}$, and NS_{KS} (see Table 1 for disaccharide structures) [20].

The aim of the current study is to develop a reproducible, fast, simple, and sensitive CZE-LIF method for the profiling and the quantification of the disaccharides derived from HS (8 major), CS/DS (8 major), HA (1), and KS (2 major) in a single CZE run and to show the applicability of this method to a biological sample that contains a similarly complex mixture of GAGs. Such a CZE-LIF method should be useful for the rapid profiling and quantification of the total GAGs in glycomics studies and improves our understanding the biological role of GAGs in biology.

Table 1 LOQ and coefficient of determination (r^2) for 19 AMAC-labeled disaccharides

Disaccharides	Abbrev.	LOQ (ng μL^{-1})	Linearity range (ng μL^{-1})	Coefficient of determination (r^2)
HS/HP disaccharides				
ΔUA(1-4)GlcNAc	$0S_{HS}$	0.1	0.1–15	0.999
ΔUA(1-4)GlcNS	NS_{HS}	0.5	0.5–20	0.999
ΔUA(1-4)GlcNS6S	$6S_{HS}$	0.1	0.1–15	0.998
ΔUA2S(1-4)GlcNAc	$2S_{HS}$	0.1	0.1–15	0.999
ΔUA(1-4)GlcNS6S	$NS6S_{HS}$	0.5	0.5–20	0.993
ΔUA2S(1-4)GlcNS	$2SNS_{HS}$	0.1	0.1–15	0.995
ΔUA2S(1-4)GlcNAc6S	$2S6S_{HS}$	0.1	0.1–15	0.997
ΔUA2S(1-4)GlcNS6S	$TriS_{HS}$	0.5	0.5–20	0.997
HA disaccharide				
ΔUA(1-3)GlcNAc	HA	0.1	0.1–15	0.999
CS/DS disaccharides				
ΔUA(1-3)GalNAc	$0S_{CS}$	0.1	0.1–15	0.999
ΔUA(1-3)GalNAc4S	$4S_{CS}$	0.1	0.1–15	0.997
ΔUA(1-3)GalNAc6S	$6S_{CS}$	0.1	0.1–15	0.996
ΔUA2S(1-3)GalNAc	$2S_{CS}$	0.1	0.1–15	0.999
ΔUA2S(1-3)GalNAc4S	SB_{CS}	0.1	0.1–15	0.999
ΔUA2S(1-3)GalNAc6S	SD_{CS}	0.1	0.1–15	0.998
ΔUA(1-3)GalNAc4S6S	SE_{CS}	0.1	0.1–15	0.999
ΔUA2S(1-3)GalNAc4S6S	$Tris_{CS}$	0.1	0.1–15	0.998
KS disaccharides				
Gal(1-4)GlcNAc6S	NS_{KS}	0.1	0.1–15	0.996
Gal6S(1-4)GlcNAc6S	$2S_{KS}$	0.1	0.1–15	0.999

ΔUA 4-deoxy-α-L-*threo*-hexenopyranosyluronic acid, *GlcN N*-acetyl glucopyransose, *GalN N*-acetylgalactopyransose, *Gal* galactopyranose, *Ac* acetyl, *S* sulfo, *LOQ* limit of quantification

Materials

Bovine corneas were from Pel-freeze Biological (Rodgers, AR, USA). Actinase E was from Kaken Biochemicals (Tokyo, Japan). Keratan sulfate (Na salt from bovine cornea) and keratanase II (from *Bacillus* sp. Ks 36) were purchased from Seikagaku Corporation (Japan). Keratanase II was dissolved in water and stored at −80 °C for up to 6 months. Unsaturated disaccharides standards of CS (0S, ΔUA-GalNAc; 4S, ΔUA-GalNAc4S; 6S, ΔUA-GalNAc6S; 2S, ΔUA2S-GalNAc; 2S4S or SB, ΔUA2S-GalNAc4S; 2S6S or SD, ΔUA2S-GalNAc6S; 4S6S or SE, ΔUA-GalNAc4S6S; and TriS, ΔUA2S-GalNAc4S6S, where S is sulfo and GalNAc is *N*-acetyl-D-galactosamine), unsaturated disaccharides standards of heparan sulfate (0S, ΔUA-GlcNAc; NS, ΔUA-GlcNS; 6S, ΔUA-GlcNAc6S; 2S, ΔUA2S-GlcNAc; 2SNS, ΔUA2S-GlcNS; NS6S, ΔUA-GlcNS6S; 2S6S, ΔUA2S-GlcNAc6S; and TriS, ΔUA2S-GlcNS6S, where GlcNAc is *N*-acetyl-D-glucosamine) and unsaturated disaccharides standard of hyaluronan (ΔUA-GlcNAc) were obtained from Seikagaku (Japan). Internal standard disaccharide ΔUA2S-GlcNCOCH$_2$CH$_3$6S (where COCH$_2$CH$_3$ in propanoyl) was from Iduron, Manchester, UK. Recombinant heparin lyase I, II, and III from *Flavobacterium heparinum* were expressed in our laboratory in *Escherichia coli* strains, provided by Professor Jian Liu (University of North Carolina, College of Pharmacy, Chapel Hill, NC, USA). The heparinases were stored at −80 °C in 10 % (*v*/*v*) glycerol for up to 6 months. Chondroitin lyase ABC from *Proteus vulgaris* and chondroitin lyase ACII from *Arthrobacter aurescens* was from Seikagaku Corporation (Tokyo, Japan). The chondroitinases were reconstituted with water and stored at −80 °C for up to 6 months. AMAC (≥98.0 %) and sodium cyanoborohydride (≥95.0 %) was supplied from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade. Vivapure Q Mini H columns were from Sartorius Stedium Biotech (Bohemia, NY, USA). Amicon ultracentrifugal filters (YM-10; 1000 molecular weight cut-off) were from Millipore (Billerica, MA, USA).

Recovery of bovine corneal GAGs

A bovine cornea was cut into the small pieces and individually subjected to proteolysis at 55 °C with 10 % (*w*/*v*) of actinase E (20 mg/mL in HPLC-grade water, Kaken Biochemicals, Tokyo, Japan) for 2 days at pH 6.0. After proteolysis, particulates were removed from the resulting solution by centrifugation at 12,000×*g* for 5 min. The supernatant was then concentrated

using Microcon YM-10 centrifugal filter units (10 kDa molecular weight cutoff, Millipore) by centrifugation at 12,000×*g* and washed with 15 ml of distilled water to remove peptides and salts. The retentate was collected and lyophilized and dissolved in 0.5 ml of 8 M urea containing 2 % CHAPS (pH 8.3) and then loaded to Vivapure Q Mini H column (Bohemia, NY, USA) equilibrated with 200 μL of 8 M urea containing 2 % CHAPS (pH 8.3) and put under centrifugal force (700×*g*). The columns were then washed with 200 μL of 8 M urea containing 2 % CHAPS at pH 8.3, followed by two washes with 200 μL of 200 mM NaCl. GAGs were released from the column by washing three times with 450 μL of 16 % NaCl and then collected eluent was desalted using YM-10 spin column (12,000×*g*). Finally, the desalted GAGs (~15.0 mg) were lyophilized.

Breakdown of GAGs to disaccharide products

A portion of the recovered GAGs from bovine cornea (~100 μg) were depolymerized using the enzyme mixture of heparinase I, II, and III (5 mU each/20 μL), chondroitinase ABC and chondroitinase AC II (5 mU each/ 20 μL), and keratanase II (5 mU/20 μL) in 100 μL 100 mM ammonium acetate (2 mM calcium chloride, pH 7.5) at 37 °C for 10 h [20]. The enzymes used to digest the 5.0 mg of GAGs were each in >50-fold excess to that required to completely digest a standard mixture of the same quantity of each GAG. The enzymatic products were then passed through the YM-10 spin columns (12,000×*g*) for 5 min then were freeze-dried for AMAC labeling reaction.

Derivatization of disaccharides with AMAC

The freeze-dried bovine corneal samples containing GAG-derived disaccharides or disaccharide standards were redissolved in 5 μL of 0.1 M AMAC in acetic acid/dimethyl sulfoxide (DMSO) (3:17, *v*/*v*) and left at room temperature for 30 min. After that, 5 μL of 1 M $NaBH_3CN$ was added to the reaction mixture and mixture was incubated at 45 °C for 4.5 h [34]. Finally, bovine cornea sample containing AMAC-labeled GAGs were diluted to various concentrations using DMSO/water (50:50; *v*/*v*), and then CE-LIF or LC-MS analysis was performed.

CZE-LIF analysis of AMAC labeled disaccharides

CZE analyses of the AMAC-labeled disaccharides were carried out HPCE system (Agilent Technologies, Wilmington, DE, USA) coupled with a ZETALIF laser induced fluorescent detector (Picometrics, Toulouse, France). All CZE experiments were performed using bare-fused silica capillary (Agilent, 50 μm i.d.×60 cm, 45 cm to detector). Each new capillary was conditioned before it was used. This conditioning procedure is necessary to ensure fully and uniformly charged capillary surface. A new capillary was rinsed with methanol (20 min), water (5 min), 1 M HCl (20 min), water (5 min), 1 M NaOH (15 min) water (5 min), 0.1 M NaOH (20 min), water (5 min), and operating buffer (10 min). The capillary was also preconditioned (optimized procedure 3) with 0.1 M HCl (3 min), 0.1 M NaOH (7 min), HPLC-grade water (4 min), and operating buffer (5 min) before each new set of runs. After each day of use, the capillary was rinsed with water for 5 min and then dried with air for 2 min to extend of the lifetime of capillary. Sodium phosphate buffer (50 mM, pH 3.3) was used as operating buffer, and the separations were carried out at 25 °C by applying 25 kV voltage. The sample was injected hydrodynamically by the application of a pressure of 50 mbar for 5 s.

HPLC-MS analysis of AMAC-labeled disaccharides

HPLC-MS analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc.) equipped with a 6300 ion trap and a binary pump. The column used was a Poroshell 120 C18 column (2.1×150 mm, 2.7 μm, Agilent) at 45 °C. Eluent A was 80 mM ammonium acetate solution and eluent B was methanol. Eluent A and 15 % eluent B were flowed (150 μL/min) through the column for 5 min followed by a linear gradient from 15 to 30 % solution B from 5 to 30 min. The column effluent entered the electrospray ionization MS source for continuous detection by MS. The electrospray interface was set in negative ionization mode with a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source temperature of 350 °C, to obtain the maximum abundance of the ions in a full-scan spectrum (150–1,200 Da). Nitrogen (8 L/min, 40 psi) was used as a drying and nebulizing gas [21].

Results and discussion

Major disaccharides were obtained by enzymatic digestion of HS/HP, CS/DS, HA, and KS. The enzymatic digestion using heparinase I, II, and III afford eight major unsaturated disaccharides for HS/HP, chondroitinase ABC, chondroitinase AC II affords eight major unsaturated disaccharides for CS/DS and one major unsaturated disaccharide for HA and keratanase II affords two major saturated disaccharides for KS [30, 20, 40] (see Table 1 for structures). The HS/HP, CS/DS, and HA disaccharides are all commercially available as standards. The KS disaccharides were prepared from purified bovine corneal KS [19, 20].

The disaccharides were next labeled with AMAC by reductive amination. The aldehyde (hemiacetal) group at the reducing end of each disaccharide is coupled with amino group of AMAC, leading to the formation of a Schiff base intermediate which is immediately reduced to a stable secondary amine linkage using sodium cyanoborohydride. The derivatization involves a simple one-step procedure and can be monitored using CE with ultraviolet detection at 232 nm or using CZE-LIF λ_{ex}=425, λ_{em}=530. Moreover, in contrast to many other labeling reagents that need to be removed before analysis, excess AMAC is not detrimental in CZE analysis [21–26, 30–36].

A number of CZE methods have previously been developed for the analysis of CS/DS or HS or HA derivative disaccharides that afford excellent resolution, efficient separation, and short analysis times. Typically, CZE can resolve these negatively charged disaccharides using either low pH operating buffers (pH 3.0 or 3.5) under reversed polarity or in high pH operating buffers (pH 8.0 to 10.4, with or without additives) under normal polarity [30–39].

Our laboratory relied on 50 mM phosphate buffer (pH 3.5) under reversed polarity at 25 kV to successfully separate AMAC-labeled HS and CS disaccharides in a single run. Under reversed polarity conditions AMAC-labeled disaccharides move through the capillary in groups, first tri-, then di-, mono-, and finally nonsulfated disaccharides. This elution order is particularly useful in investigating the sulfation patterns of GAGs present in biological samples. GAG-derived disaccharides contain different negative charge density and polarity due to their anionic carboxyl and *O*-sulfo and *N*-sulfo groups. CZE can also separate different disaccharides having identical net charge (i.e., $TriS_{HS}$ and $TriS_{CS}$) due to differences in their molecular shapes. The nonsulfated disaccharides move slowly in the capillary and define the length of time required for disaccharide analysis [31]. KS, the least studied of the GAGs, has structural similarities to chondroitin sulfates but also contains the unique feature of having a galactopyranose residue instead of a pyranosyluronic acid residue. While there have been some efforts to separate CS and KS GAGs using agarose-gel electrophoresis and size exclusion chromatography it is often quite difficult to separate and analyze mixtures of these two GAGs [44, 45].

We next turned our attention to the simultaneous determination of the AMAC derivatives of KS, HS/HP, CS/DS, and HA disaccharides. Their simultaneous analysis was first attempted using CZE-LIF method (50 mM phosphate buffer,

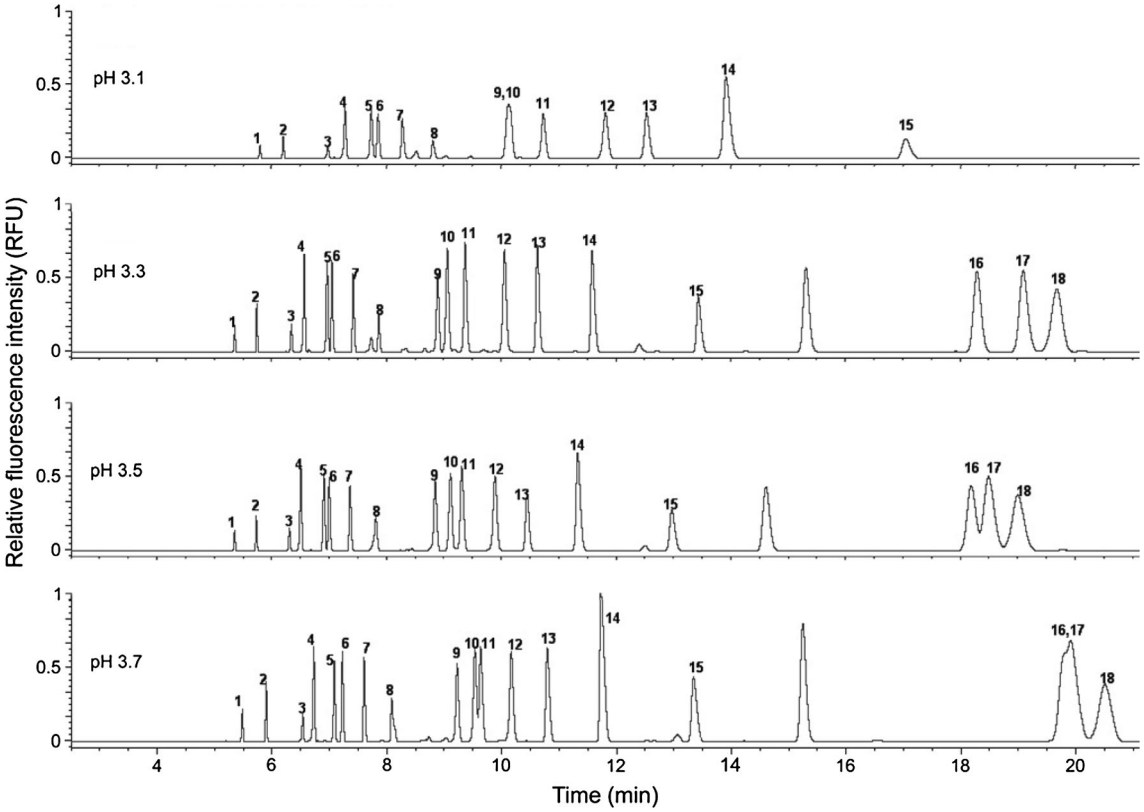

Fig. 1 Effect of phosphate buffer pH (3.1, 3.3, 3.5, 3.7) on electropherograms of disaccharides derived from HS, CS/DS, HA and KS. $TriS_{CS}$ (**1**), $TriS_{HS}$ (**2**), SD_{CS} (**3**), SB_{CS} (**4**), SE_{CS} (**5**), $2S6S_{HS}$ (**6**), $NS2S_{HS}$ (**7**), $NS6S_{HS}$ (**8**), $2S_{CS}$ (**9**), $2S_{KS}$ (**10**), $6S_{CS}$ (**11**), $4S_{CS}$ (**12**), $2S_{HS}$ (**13**), $6S_{HS}$ (**14**), NS_{HS} (**15**), NS_{KS} (**16**), $0S_{CS}$ (**17**), and HA (**18**). Excess AMAC migrates as a peak between NS_{HS} (**15**) and NS_{KS} (**16**). The concentration of disaccharide mixture is 5 ng μL^{-1}. Operating conditions in CZE-LIF: 50 mM phosphate buffer (pH 3.1, 3.3, 3.5, 3.7) in reversed polarity at 25 kV, injection time 5 s (50 mbar)

pH 3.5, under reversed polarity at 25 kV) previously developed for separating HS/HP and CS/DS disaccharides [30]. Although baseline separation of disulfated KS disaccharide (**10**) from disulfated CS disaccharides (**9**, **11**) was achieved under these operating conditions, the resolution between nonsulfated CS (**17**), and monosulfated KS (**16**) was not satisfactory (R_s=0.95). Several parameters, including pH and ionic strength of the operating buffer were next investigated to improve the resolution.

First, the impact of the phosphate buffer pH on resolution was investigated. The pK_a values of GAG derivative disaccharide carboxyl groups vary slightly from one another, therefore, small differences in the pH of operating buffer close to their pK_a values can impact the separation of HS and CS disaccharides [36]. For example, the control of buffer pH can reduce the time required for the separation of CS and HS disaccharides. The electropherograms of AMAC-labeled disaccharides over the pH range from 3.1 to 3.7 are shown in Fig. 1. As the pH increased, the resolution between monosulfated KS disaccharide (**16**) and nonsulfated CS disaccharide (**17**) decreased with both disaccharides completely overlapped at pH 3.7. Also, as the pH increased the disulfated KS disaccharide (**10**) moved between being completely overlapped with $2S_{CS}$ disaccharide (**9**) at pH 3.1 to being completely overlapped with $6S_{CS}$ disaccharide (**11**) at pH 3.7. These results show that buffer pH is one of the critical parameters for resolving AMAC-labeled KS and CS disaccharides. Therefore, pH 3.3, giving the optimal separation within a 30 min period, was selected for further studies. It is noteworthy that peak intensity changes with changing pH, which is consistent with previously reported data on pH-mediated stacking of anions in CE [46].

Next, the effect of the ionic strength of the operating buffer on separation was examined by varying the buffer concentration from 30 to 60 mM (Fig. 2). The results of these experiments demonstrated that operating buffer concentration contributed little, except in the resolution between SE_{CS} (**5**) and $2S6S_{HS}$ (**6**). Resolution between these two disaccharides was optimal using 50 mM operating buffer, which was selected as buffer concentration for all further experiments. Thus, by using 50 mM phosphate buffer (pH 3.3) under reversed polarity at 25 kV the 19 AMAC-labeled disaccharides derived from HS/HP, CS/DS, HA and KS could be baseline-separated in less than 25 min.

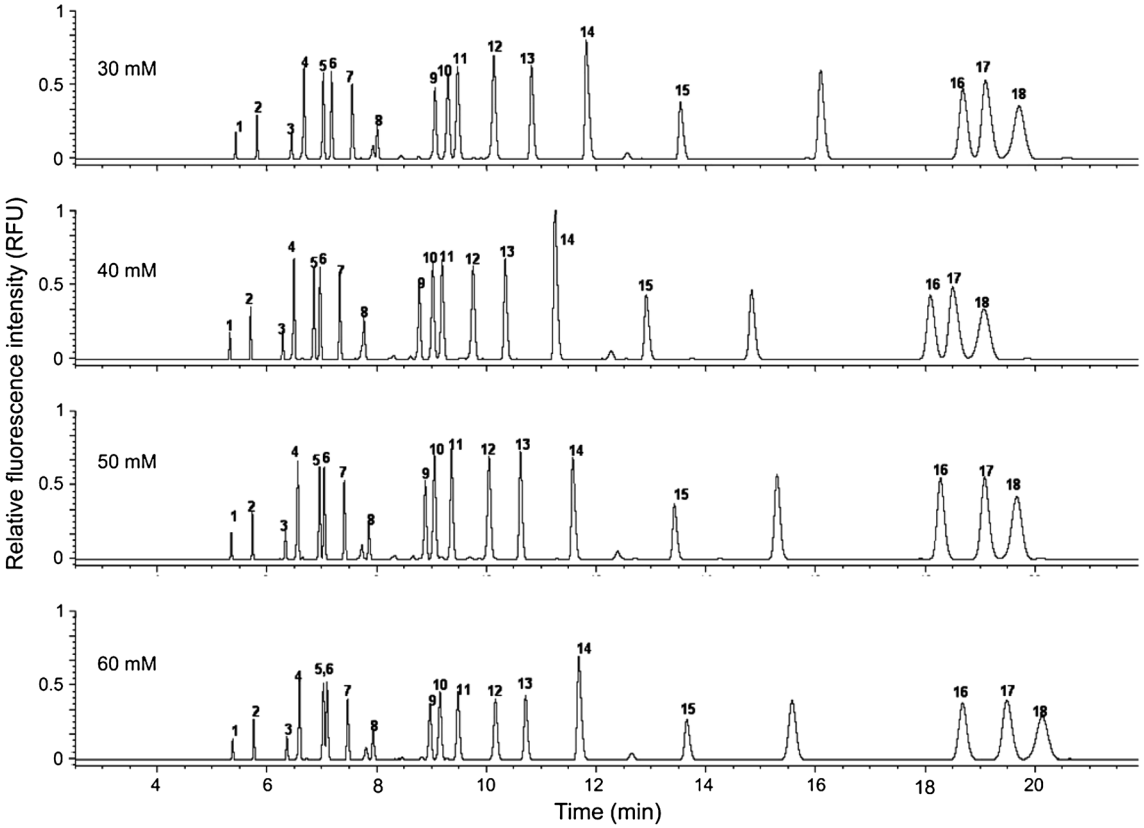

Fig. 2 Effects of phosphate buffer concentration on CZE-LIF separation of disaccharides derived from HS, CS/DS, HA, and KS. $TriS_{CS}$ (**1**), $TriS_{HS}$ (**2**), SD_{CS} (**3**), SB_{CS} (**4**), SE_{CS} (**5**), $2S6S_{HS}$ (**6**), $NS2S_{HS}$ (**7**), $NS6S_{HS}$ (**8**), $2S_{CS}$ (**9**), $2S_{KS}$ (**10**), $6S_{CS}$ (**11**), $4S_{CS}$ (**12**), $2S_{HS}$ (**13**), $6S_{HS}$ (**14**), NS_{HS} (**15**), NS_{KS} (**16**), $0S_{CS}$ (**17**), and HA (**18**). Excess AMAC migrates as a peak between NS_{HS} (**15**) and NS_{KS} (**16**). The concentration of disaccharide mixture is 5 ng μL^{-1}. Operating conditions in CZE-LIF—30 40, 50, 60 mM phosphate buffer (pH 3.3) in reversed polarity at 25 kV, injection time 5 s (50 mbar)

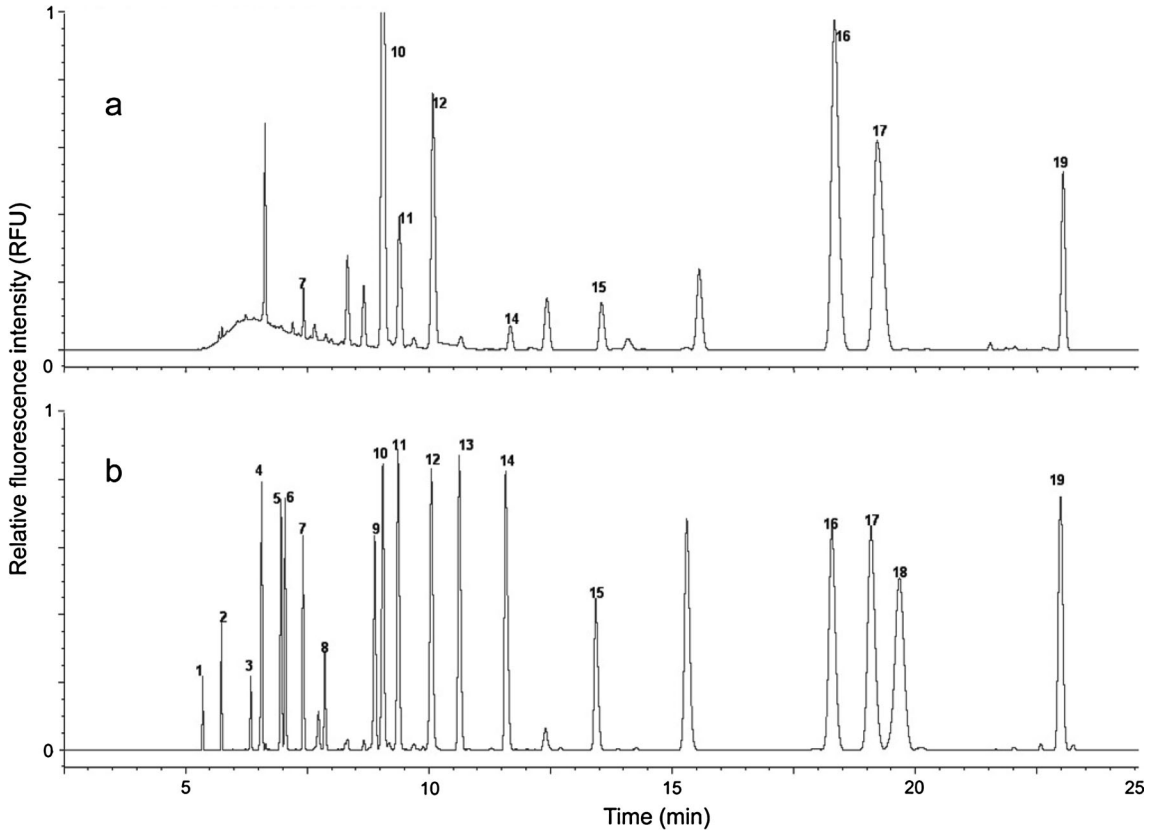

Fig. 3 Electropherograms of a bovine cornea sample (**a**) and disaccharide standard mixture (**b**) (5 ng μL^{-1} for each disaccharide). $TriS_{CS}$ (**1**), $TriS_{HS}$ (**2**), SD_{CS} (**3**), SB_{CS} (**4**), SE_{CS} (**5**), $2S6S_{HS}$ (**6**), $NS2S_{HS}$ (**7**), $NS6S_{HS}$ (**8**), $2S_{CS}$ (**9**), $2S_{KS}$ (**10**), $6S_{CS}$ (**11**), $4S_{CS}$ (**12**), $2S_{HS}$ (**13**), $6S_{HS}$ (**14**), NS_{HS} (**15**), NS_{KS} (**16**), $0S_{CS}$ (**17**), HA (**18**), and $0S_{HS}$ (**19**). Operating conditions in CZE-LIF: 50 mM phosphate buffer (pH 3.3) in reversed polarity at 25 kV, injection time 5 s (50 mbar)

Reproducibility of the CE-LIF method

Fresh operating buffer was used for each analysis to prevent from buffer depletion and also preconditioning step was applied to capillary before each analysis. This afforded reproducible migration times for 19-AMAC labeled disaccharides in the CZE. A preconditioning step between consecutive runs was necessary to achieve reproducible migration times. This preconditioning step cleanses and equilibrates the capillary surface and ensures a consistent electroosmotic flow. Three different preconditioning procedures were investigated to improve the reproducibility of the migration time of 19-AMAC

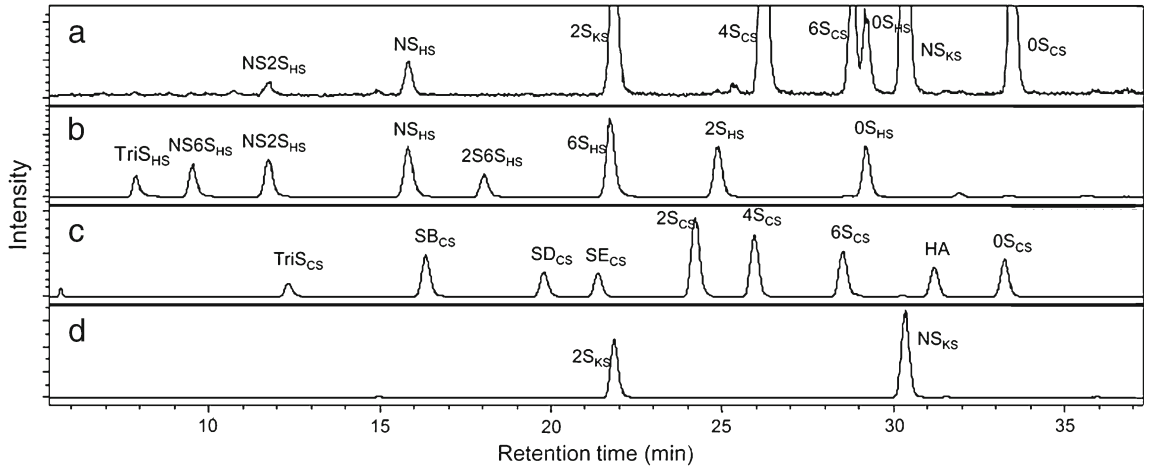

Fig. 4 LC-MS chromatogram of a bovine cornea sample (**a**) and HS (**b**), CS (**c**), and KS (**d**) disaccharide standards

labeled disaccharides. The results show that preconditioning procedures 2 and 3 gives the similar relative standard deviation (RSD) value for some disaccharides ($2S6S_{HS}$, $NS2S_{HS}$, $2S_{HS}$, NS_{HS}, SB_{CS}, $4S_{CS}$, $2S_{KS}$). However, preconditioning procedure 3 resulted in the best migration time reproducibility for all disaccharides except NS_{KS} but the RSD value for this disaccharide is very reasonable (0.6 %) (see Electronic supplementary material Fig. S1). Under these conditions, the reproducibility of migration times for each disaccharide gives a RSD of ≤1.03 %. Also, interday reproducibility of migration times was found between 2.3 and 4.4 % using the same capillary and under the same operating conditions.

Intraday and interday reproducibility of the CZE-LIF method were also evaluated at three different concentrations (1, 5, 15 ng μL^{-1}) for each disaccharide and the interday reproducibility experiments were performed over five consecutive days. The results show that the intraday reproducibility did not exceed 5.6 %, while the interday reproducibility varied between 4.15 and 8.69 %.

Response linearity was determined using six concentrations and a calibration curve was constructed by plotting the peak area of disaccharides as a function of disaccharide concentration. The limit of quantification (LOQ) and correlation for the determination of each disaccharide is shown in Table 1. The calibration curve had a coefficient of determination (r^2) value greater than 0.993, which indicates a linear fit between disaccharide concentration and response.

Analysis of GAGs composition (HS, CS/DS, HA, and KS) in bovine cornea

The applicability of this CZE-LIF method to a biological sample was next examined by analyzing the bovine corneal tissue samples. GAGs were first extracted from bovine cornea. Cornea was selected, as it is one of the few tissues known to be relatively rich in KS [47]. We followed an extraction procedure established in our laboratory for the quantitative recovery of GAGs from cells, tissues (including cornea), and biological fluids, which relies on the use of strong anion exchange (SAX) spin columns [48, 49]. After extraction of GAGs from bovine cornea, GAGs were digested with the mixture of heparinase I, II, and III, chondroitinase ABC, chondroitinase ACII, and keratinase II. The resulting disaccharides were AMAC-labeled and analyzed using our CZE-LIF method (Fig. 3). The results show that bovine cornea contained 9 different disaccharides: $0S_{HS}$ (**19**), NS_{HS} (**15**), $6S_{HS}$ (**14**), $2SNS_{HS}$ (**7**), $4S_{CS}$ (**11**), $6S_{CS}$ (**12**), $0S_{CS}$ (**17**), NS_{KS} (**16**), and $2S_{KS}$ (**10**). The weight percentages of these disaccharides was determined to be 0.8, 0.7, 0.1, 0.6, 5.9, 3.2, 5.1, 11.7, and 71.8 %, respectively. We had initially used external standard calibration to quantify the disaccharides in bovine cornea. Next, the use of an internal standard calibration was examined. A major issue encountered in using internal standard calibration curve for the disaccharide analysis is associated with finding the right internal calibrant. We first examined an AMAC-labeled unnatural disaccharide, ΔUA2S–GlcNCOEt6S, as internal standard, but it comigrated with other AMAC-labeled disaccharides interfering with our analysis. Ultimately, we decided to use one of the disaccharides missing in the bovine cornea, AMAC-labeled $TriS_{cs}$, as our internal standard. The results obtained using a calibration curve based on internal standard was not significantly different ($p>0.05$) from our external calibration curve. To further validate this analysis, the same sample was also analyzed by LC-MS (Fig. 4). The results showed that LC-MS and CZE-LIF methods gave comparable results, suggesting that the proposed CZE-LIF method can be accurately applied for the total GAG determination of bovine cornea. In addition to the use of an external standard calibration curve, an internal standard or standard addition method can be used for the identification and quantification of disaccharides. Standard addition methods such as spiking an authentic disaccharide standard into the sample is particularly helpful for the identification of closely migrating disaccharides in complex biological samples.

Conclusions

A fast, reproducible, simple, and sensitive CZE-LIF method was developed for the simultaneous profiling and quantification of major disaccharides derived from HS, CS/DS, HA, and KS in biological samples. This CZE-LIF method afforded the complete separation of 19 AMAC-labeled disaccharides within 25 min. This CZE-LIF method when applied to determination of total GAGs in bovine cornea gave results that compared well with LC-MS analysis. On the basis of these results, we propose the use of CZE-LIF for analysis of total GAG amounts in cornea as well as in the other tissues.

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