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REVIEW

Capillary electrophoresis for the analysis of glycosaminoglycans and glycosaminoglycan-derived oligosaccharides

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ABSTRACT: Glycosaminoglycans are a family of polydisperse, highly sulfated complex mixtures of linear polysaccharides that are involved in many life processes. Defining the structure of glycosaminoglycans is an important factor in elucidating their structure–activity relationship. Capillary electrophoresis has emerged as a highly promising technique consuming an extremely small amount of sample and capable of rapid, high-resolution separation, characterization and quantitation of analytes. Numerous capillary electrophoresis methods for analysis of intact glycosaminoglycans and glycosaminoglycan-derived oligosaccharides have been developed. These methods allow for both qualitative and quantitative analysis with a high level of sensitivity. This review is concerned with separation methods of capillary electrophoresis, detection methods and applications to several aspects of research into glycosaminoglycans and glycosaminoglycan-derived oligosaccharides. The importance of capillary electrophoresis in biological and pharmaceutical samples in glycobiology and carbohydrate biochemistry and its possible applications in disease diagnosis and monitoring chemical synthesis are described.

INTRODUCTION

Structural characteristics of glycosaminoglycans

Glycosaminoglycans (GAGs) are a family of highly sulfated, complex, polydisperse linear polysaccharides that display a variety of important biological roles (Linhardt and Toida, 1997; Lane and Lindahl, 1989). The structural complexity is further compounded by their sequence heterogeneity, caused primarily by a variation of degree and position of sulfate groups. They can be categorized into three main structural groups: heparin/heparan sulfate group, dermatan/chondroitin sulfate group and hyaluronan. GAGs composed of the disaccharide repeating units are shown in Fig. 1.

Heparin and heparan sulfate are structurally related and comprise a hexouronic acid residue (either L-iduronic or D-glucuronic acid) $1 \rightarrow 4$ linked to a D-glucosamine (either *N*-sulfo or *N*-acetyl-D-glucosamine) residue. The major repeating unit in heparin is →4-α-D-*N*-sulfoglucosamine-6-sulfate $(1 \rightarrow 4)$-α-L-iduronic acid-2-sulfate (1→, corresponding to 75–90% of its sequence, whereas heparn sulfate consists of 50–75% $\rightarrow$ 4-α-D-*N*-acetyl-glucosamine $(1 \rightarrow 4)$-β-glucuronic acid (1→ and smaller amounts of →4-α-D-*N*-acetyl-glucosamine-6-sulfate (1→)-β-D-glucuronic acid (1→ and →4-α-D-*N*-sulfoglucosamine$(1 \rightarrow 4)$-β-D-glucuronic acid (1→. Heparin, which contains approximately 2.7 sulfate groups per disaccharide unit, is more highly sulfated than heparan sulfate, which contains less than one sulfate per disaccharide unit. Structural studies clearly indicate that the structures of heparin and heparan sulfate are distinctly different. Heparin is composed of a major trisulfated disaccharide repeating unit, but it also contains a number of additional disaccharide structures (Linhardt *et al.*, 1988; Loganathan *et al.*, 1990; Pervin *et al.*, 1995; Linhardt and Loganathan, 1990). It is these additional disaccharide units that make heparin's structure complex and that also comprise the antithrombin (AT) pentasaccharide binding site, important for heparin's anticoagulant activity.

The dermatan/chondroitin sulfate are the most common type of GAGs in extracellular matrix proteoglycans (Vogel, 1994), which comprises alternating uronic acid and *N*-acetyl-galactosamine residues (Gunay and Linhardt, 1999; Silbert, 1996). Chondroitin sulfate A consists of unsulfated D-glucuronic acid $1 \rightarrow 3$ linked to *N*-acetylated, 4-sulfated D-galactosamine, which in turn is attached to the next D-glucuronic acid residue by a $1 \rightarrow 4$ linkage. Chondroitin sulfate C has a 6-sulfo group

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Abbreviations used: AT, antithrombin; GAG, glycosaninoglycan; LMW, Low Molecular Weight.

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on the *N*-acetylated D-galactosamine in place of the 4-sulfo group found in chondroitin sulfate A. Dematan sulfate is similar to chondroitin sulfate A but instead of D-glucuronic acid it consists mainly of L-iduronic acid.

Hyaluronic acid has alternating repeating units of the structure $(1 \rightarrow 3)$ β-*N*-acetyl-D-glucosamine $(1 \rightarrow 4)$ β-D-glucuronic acid $(1\rightarrow$. Hyaluronic acid is both less highly charged and considerably larger than the other GAGs, having an average molecular weight of up to 2×10^6 Da. Hyaluronic acid is an extended, flexible, helical rod-shaped molecule. It exhibits a number of secondary structures maintained by intramolecular hydrogen bonding (Trimm and Jennings, 1983; Scott, 1992).

Biological activities of glycosaminoglycans

GAGs are synthesized by nearly all animal cells. Hyaluronic acid, chondroitin sulfate and heparan sulfate are also synthesized in prokaryotes. With the exception of hyaluronic acid, GAGs are biosynthesized as proteoglycans, attached to a protein core. GAGs are highly charged polyanions that interact with hundreds of different proteins (Linhardt and Toida, 1997). GAGs bind to proteins primarily through the interaction of their sulfo and carboxyl groups with basic amino acid residues present in shallow pockets or on the surface of GAG-binding proteins (Linhardt and Toida, 1997). In the past decade GAGs have been shown to play a role in the regulation of a large number of important cellular processes including cell growth and cell–cell interaction (Linhardt and Toida, 1997; Lane and Lindahl, 1989).

Heparin has been widely used as an anticoagulant drug for over 60 years (Linhardt, 1991; Linhardt and Toida, 1997; Linhardt and Gunay, 1999). Heparin and heparan sulfate are among the strongest acids present in the body and under physiologic conditions are highly charged polyanions (Linhardt and Toida, 1997). Virtually any cationic protein is capable of binding heparin or heparan sulfate under physiological conditions and the activity of these proteins is often modified by the binding (Linhardt and Toida, 1997). Even anionic proteins are capable of interacting with heparin and heparan sulfate. Heparin is an important activator of serine protease inhibitor (SERPIN; Linhardt, 1991). It has been also shown to regulate cellular processes through binding, stabilizing and activating growth factors leading to an increased interest in its structure (Linhardt *et al.*, 1990). Heparan sulfates have been identified in all animal tissues, where they are located mainly at cell-surface membranes or in the extracellular matrix (Giuffre *et al.*, 1997; Wudunn and Spear, 1989; Caughey *et al.*, 1994; Nakajima *et al.*, 1983; Parish *et al.*, 1987; Rosenberg *et al.*, 1997). Heparan sulfate has polysaccharide components that bind AT (Hoppensteadt *et al.*, 1989; Marcum and Rosenberg, 1984), supporting blood flow across the vascular endothelium. Heparan sulfate is involved in cell–cell interaction, and may function as the endogenous inhibitor of smooth muscle cell proliferation (Wright *et al.*, 1988) and a regulator of growth factors (Walker *et al.*, 1994). Heparan sulfate does not delay the activation of prothrombin but expresses its anticoagulant activity by catalyzing the inhibition of thrombin as it is formed in plasma. Low molecular weight (LMW) heparins have been introduced as heparin substitutes and have reduced side effects, more predictable pharmacological action, sustained antithrombotic activity, and better bioavailability. These advantages have led to the development of several commercial LMW heparin preparations.

The dermatan sulfate (Linhardt and Hileman, 1995) and chondroitin sulfate (Watanabe *et al.*, 1998) proteoglycans are also commonly found in the extracellular environment and demonstrate a multiplicity of biological functions (Bernfield *et al.*, 1999; Iozzo, 1998). Chondroitin sulfates are important in cell–cell communication (Hardingham and Fosang, 1992) and are also involved in the complement cascade and the coagulation cascade (Edens *et al.*, 1993). Dermatan sulfate exhibits venous antithrombotic activity and has been studied clinically. Dermatan sulfate's antithrombotic activity results from its acceleration of heparin cofactor II mediated inhibition of thrombin (Linhardt and Hileman, 1995; Linhardt *et al.*, 1991a,b). Dermatan sulfate resembles heparan sulfate/heparin in that it contains structural domains (Maruyama *et al.*, 1998; Forsberg *et al.*, 1991) dominated by the placement of iduronic acid-rich regions that may be important in specific interaction with proteins (Silbert, 1996; Stringer and Gallagher, 1997; Lyon *et al.*, 1998). The flexible features and unique conformation of the iduronic acid residue make dermatan sulfate a particularly interesting target for GAG–protein interaction studies. Some interaction specificity has been demonstrated for these GAGs, with heparin cofactor II, the serine proteinase inhibitor (Liaw *et al.*, 1999; Pavao *et al.*, 1998), annexins (Ishitsuka *et al.*, 1998) and other proteins (Lyon *et al.*, 1998; Scott, 1996; Petersen *et al.*, 1999; Ehnis *et al.*, 1996).

Hyaluronic acid is a prominent component of soft connective tissue and is a component of the extracellular matrix which maintains water balance and is important in cellular interactions (Laurent and Fraser, 1992). Hyaluronic acid also plays a crucial role during development and differentiation and has many other important cell regulatory activities (Laurent and Fraser, 1992; Shinomura *et al.*, 1999; Spicer and McDonald, 1998). Hyaluronic acid forms the ligand structure for a family of aggregating proteins, the hyaladherins. Hyaluronic acid also has unique rheological properties, and can be used pharmaceutically to relieve pain in osteoarthrosis and in eye surgery. Hyaluronic acid is known to interact with a variety of different proteins including link protein and CD44 (Ehnis *et al.*, 1996; Day, 1999).

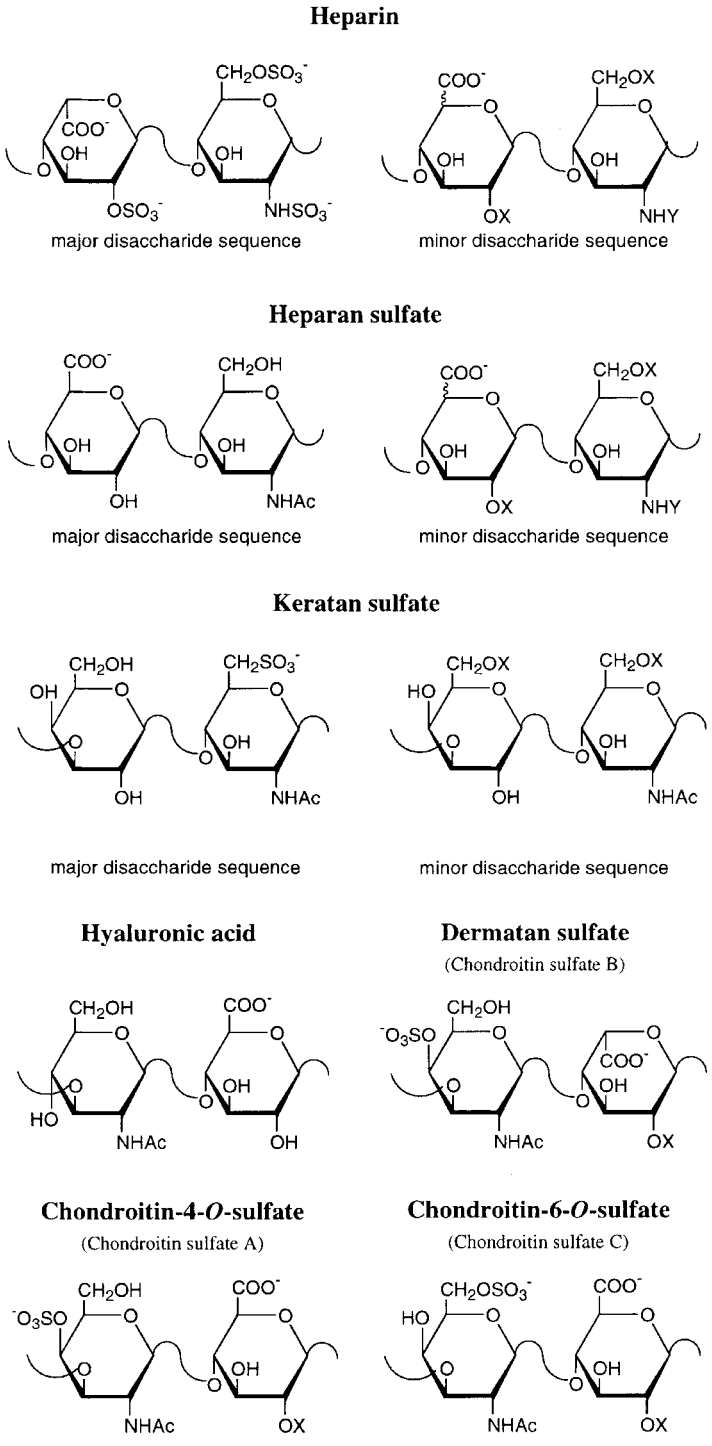

Figure 1. The structures of the major disaccharide repeating unit found in each glycosaminoglycan. Heparin, heparan sulfate and keratan sulfate have diverse sequences where $X = SO_3^-$ or H and $Y = SO_3^-$, Ac or H.

Importance of capillary electrophoresis for the analysis of GAGs and GAG-derived oligosaccharides

The structural studies of GAGs and GAG-derived oligosaccharides represent a major methodological challenge in terms of sample complexity. In the case of oligosaccharides isolated from biological samples, structural analysis must often be carried out with limited

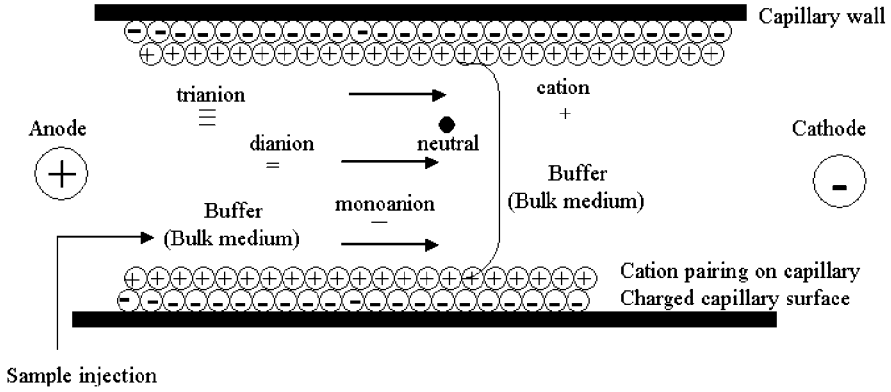

Figure 2. Separation of charged and neutral species by normal polarity mode of capillary electrophoresis. The electroosmotic flow is shown by arrow.

amount of analyte. A number of efforts have been made to develop new methods for GAG analysis.

In the last few years, capillary electrophoresis (CE) has proved to be a very attractive alternative separation technique for GAGs and GAG-derived oligosaccharides. CE affords high resolving power and great flexibility in the separation order. CE also has several advantages over a variety of other analytical methods, including an extremely high separation efficiency, on-line detection, simple operation, short analysis time, automated and reproducible analysis, and very low consumption of samples and buffers (Linhardt and Pervin, 1996).

CE analysis of intact GAGs may provide useful information on the amount, the molecular size, polydispersity and the charge density of the chain (Grimshaw *et al.*, 1996; Hayse *et al.*, 1997; Stefansson and Novotny, 1994a) and it has been used to control the purity of isolated GAG species (Malsch and Harenberg, 1996). CE at low pH can easily be used to separate and characterize intact GAGs in respect of the average number of sulfates present per repeating unit. CE has been widely applied to the disaccharide compositional analysis of GAGs (Linhardt *et al.*, 1993; Linhardt *et al.*, 1994). Detailed structural studies of GAGs also require that structures be assigned to all the distinct species resolved by CE. Conducting binding studies on immobilized oligosaccharides would also lead to a better understanding of structure–activity relationships. In addition, CE can be used to identify the purity of the GAG-derived oligosaccharides. Application of CE to the separation of GAG-derived oligosaccharides has led to significant advances in the analysis of GAG structure by CE (Linhardt, 1994a; Linhardt and Pervin, 1996; Hileman *et al.*, 1998; Kuhr, 1993; Karger *et al.*, 1989).

This review focuses on separation approaches of CE, detection methods and its applications to several aspects of research in GAGs and GAG-derived oligosaccharides. The importance of CE in biological and pharmaceutical samples in glycobiology and carbohydrate biochemistry and its possible applications to disease diagnosis and monitoring chemical synthesis are described.

SEPARATIVE PRINCIPLES OF CE

CE is a high-resolution analytical technique that has been applied to a wide variety of different types of molecules. CE separates analytes under the influence of an applied electrical field as they migrate in a narrow capillary column spanning two buffer reservoirs and filled with a conducting buffer solution (Linhardt and Pervin, 1996). The capillary column is made of fused silica, and invariably carries negative charge on its inner surface because of the presence of silanol groups. At a high voltage, the positive buffer ions migrate toward the cathode, and a narrow capillary provides a flat flow profile due to electroosmotic flow. If a sample containing both ionic and non-ionic analytes is introduced into the capillary at the anode side, electroosmotic flow drives all species from the injector through the capillary and into the detector. The positive species elute first, followed by the neutral and later the negative species, thus effecting a separation. This electroosmotic flow effects the mobility of analytes through the capillary. Separation is optimized by manipulating the electroosmotic flow (Linhardt and Pervin, 1996). The rate of electroosmotic flow depends on the pH of the operating buffer. The positive ions (anode) have their electrophoretic mobility in the same direction as the electroosmotic flow. The mobility of anion electrophores depends on the net difference between the electroosmotic flow and electrophoretic mobility. The two different modes, normal polarity and reversed polarity, of CE are widely used to analyze GAGs and GAG-derived oligosaccharides.

Normal polarity mode is the most common mode of separation by CE (Fig. 2). In normal polarity, the sample is injected at the anode and detected at the cathode and basic or neutral buffer is required. Negatively charged

species are prevented from migrating out of the capillary by the dominant force of electroosmotic flow. At pH above 3.0, the silanol residues on the capillary wall are ionized yielding a negatively charged inner surface of the capillary. Positive ions in the buffer electrolyte are attracted to those silanol residues. Thus, under these conditions the electroosmotic flow is sufficient to permit the use of normal polarity even when separating anions by CE. Normal polarity separations in basic borate buffer containing sodium dodecyl sulfate appear to give the best separation of mixtures of higher oligasaccharides (Pervin *et al.*, 1994b). While organic solvent additives, such as formamide, can improve these separations, such additives have an adverse impact on the sensitivity of detection (Desai *et al.*, 1993a).

In reversed-polarity mode, the sample is applied at the cathode and detected at the anode, and an acidic buffer is required. At very low pH (<3), the silanol residues on the capillary wall lose their negative charge, thus the electroosmotic flow is decreased. At acidic pH, the electroosmotic flow is too weak to overcome the electrophoretic mobility. The major force in the separation is the mobility of ions under electrophoresis. Reversed-polarity CE has been successful in low pH analysis of a variety of GAG-derived oligosaccharides (Pervin *et al.*, 1994b).

The resolution achieved by CE under a given set of conditions is dependent mainly on the charge, mass and molecular mobility of the analytes present. Detection is typically by ultraviolet (UV) absorbance or fluorescence emission (Karger *et al.*, 1989; Linhardt, 1994a; Linhardt *et al.*, 1993).

MEANS OF DETECTION IN CE ANALYSIS OF UNDERIVATIZED GAGs AND GAG-DERIVED OLIGOSACCHARIDES

Direct UV detection

UV detection is by far the most versatile detection method in CE, and is implemented in every commercial CE system. Direct UV detection use for the analysis of intact GAGs is restricted, however, because of their lack of conjugated π-systems and consequently the extremely low extinction coefficients (Grimshaw *et al.*, 1994, 1996; Malsch *et al.*, 1996). The GAG-derived oligosaccharides show characteristic absorption with $\lambda = 232$ nm at micromolar concentrations due to the unsaturated uronic acid ($\Delta^{4,5}$-uronic acid) residue present at the non-reducing termini of these molecules (Karamanos *et al.*, 1995; Al-Hakim *et al.*, 1991; Ampofo *et al.*, 1991; Honda *et al.*, 1992). The disadvantage of methods that use the formation and detection of unsaturated uronic acid terminated oligosaccharides is that the sensitivity depends on molecular weight. This results in a limit of detection of a femtomole of oligosaccharide on commercially available absorbance detectors. These UV wavelengths limit the selection of buffers and additives. Trace impurities can often interfere with such analysis (Grimshaw, 1997).

Indirect UV detection

CE with indirect UV detection is an area of development to overcome the difficulty encoutered with the analysis of carbohydrates that lack chromophores in their structures (Grimshaw, 1997). The detection principle is based on the displacement of the chromophore in the background electrolyte by the analyte molecule, resulting in negative peaks. To ensure adequate detection limits, a carrier electrolyte anion with a high molar absorptivity and an effective electrophoretic mobility close to the mobilities of the analytes is required (Paule and Klockow, 1996). The buffers of benzene-1,2,4,-tricarboxylic acid and of 5-sulfosalicylic acid are generally used (Damm and Overklift, 1994). Low detection limits can be achieved with carbohydrates carrying intrinsic negative charges (Damm and Overklift, 1994; Bergholdt *et al.*, 1993). Indirect UV detection has been applied to the GAGs using buffers with pH optimization for baseline resolution of peaks. This approach gives an order of effective increase in sensitivity over the direct absorption method and response factors are almost independent of the GAG structure (Grimshaw, 1997).

Detection as a metal complex

Sensitive detection of intact GAGs is now possible utilizing copper complexes (Wiley, 1995; Toida and Linhardt, 1996; Toida *et al.*, 1997). GAG polymers form colored complexs with Cu (II) ions in a copper sulfate solution. The CE analysis of the copper (II)–GAG complexes is performed by reversed-polarity electrical field gradient at a migration buffer of pH 3.0, and detection is achieved at 240 nm. Under optimized conditions, each GAG shows a broad, featureless peak having a distinctive migration time. The sensitivity of this method towards GAG polymers varies greatly with the structure of the substrate (Toida and Linhardt, 1996; Toida *et al.*, 1997b).

MS detection

CE interfaced with structural information producing detection systems such as mass spectrometry (MS) represents invaluable tools in GAGs analysis. On-line CE-MS has been performed using electrospray ionization–mass spectrometry (ESI-MS), continuous-low fast atom bombardment ionization sources and the time-of-flight mass spectrometer (TOF-MS; Niessen *et al.*, 1993; Lazar *et al.*, 1997). Electrospray appears to be an ideal

source for CE, producing mainly the protonated molecular ion for LMW compounds, thus making possible the MS analysis of analytes with molecular weights in the range of millions. TOF-MS has been evaluated as a detector for fast CE that provides excellent detection in the very low femtomole range and attomole range (Lazar *et al.*, 1997). A direct coupling between CE and ionspray mass spectrometry has been optimized to identify oligosaccharides obtained by enzymatic digestion of heparin (Duteil *et al.*, 1999). Although the different combinations of CE polarity and MS ionization mode can provide complementary information, the use of positive polarity CE with negative MS ionization seems to be the best approach to analyze complex oligosaccharide mixtures (Duteil *et al.*, 1999).

MS detection not only improves the sensitivity compared to CE alone, but also provides high resolution of the different core glycoforms and oligosaccharides (Auriola *et al.*, 1998). The additional structural data obtained by MS decreases reliance on precise retention migration time measurements required by other methods that involve separations based solely on electrophoresis (Bazin *et al.*, 1999). It can be anticipated that CE/MS will become a powerful tool for the characterization of complex GAGs (Niessen *et al.*, 1993).

CE ANALYSIS OF UNDERIVATIZED GAGs

Hyaluronan

Despite considerable molecular polydispersity, procedures for the separation and identification of hyaluronic acid by CE are well established. Intact hyaluronic acid migrates in untreated fused-silica capillaries as a single broad peak at a buffer of phosphate borate, pH 9.0, containing sodium dodecyl sulfate, and normal polarity (Grimshaw *et al.*, 1994, 1996). The hyaluronic acid from vitreous humor has been analyzed by this method, using direct UV detection (Grimshaw *et al.*, 1994). Hyaluronic acid in synovial fluid from joints in a state of rheumatoarthritic disease may migrate in the same run buffer as a very broad peak or a group of overlapping broad peaks, but migration of hyaluronic acid is affected by the interaction with proteins. Adding sodium dodecyl sulfate may decrease such interactions (Grimshaw *et al.*, 1994). Hyaluronic acid samples with different molecular weight have been quantitatively determined by CE in a bare fused silica capillary using a slightly acidic buffer (pH 4.0) as the running buffer. Hyaluronic acid under weakly acidic conditions and reversed polarity migrated as a narrower peak. Calibration curves showed good linearity with similar slopes from 0.01 to 3.3 mg/mL for hyaluronic acid samples with different molecular mass distributions. When pullulan was added to the operating buffer, the migration was affected in a manner dependent on the molecular size (Hayse *et al.*, 1997). Detection of intact hyaluronic acid is possible utilizing copper complexes (Toida and Linhardt, 1996) by reversed-polarity CE, but compared to other copper (II)–GAG complexes, hyaluronic acid gives low detection sensitivity. Hyaluronan oligomers can be separated on a capillary filled with polyacrylamide gel and the sequence shows a linear relationship between migration times and degree of polymerization (Novotny and Sudor, 1993). Hyaluronic

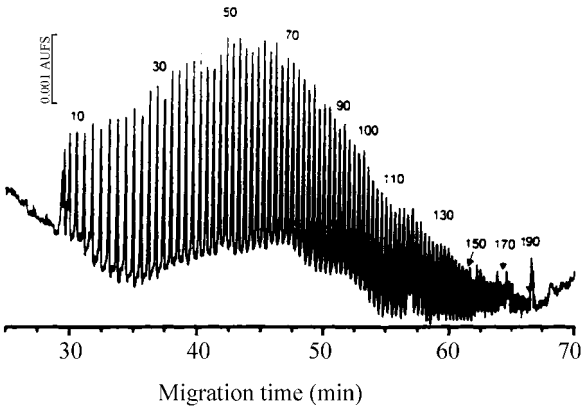

Figure 3. Separation of hyaluronic acid polymers. The numbers indicate the degree of polymerization. Analytical conditions: capillary, fused-silica capillary coated with (50% phenyl) methylpolysiloxane (57 cm × 100 μm i.d.; effective length, 50 cm); buffer, 0.1 M Tris–0.25 M borate (pH 8.5) containing PEG 70 000 at 10%; applied voltage, 10 kV (6.2 μA). The sample solution (1 mg/mL) was introduced for 10 s at 5 kV by the electrokinetic method. (Reprinted from *Anal. Chem.* 1999; **71**: 1592–1596. Copyright © 1999 American Chemical Society.)

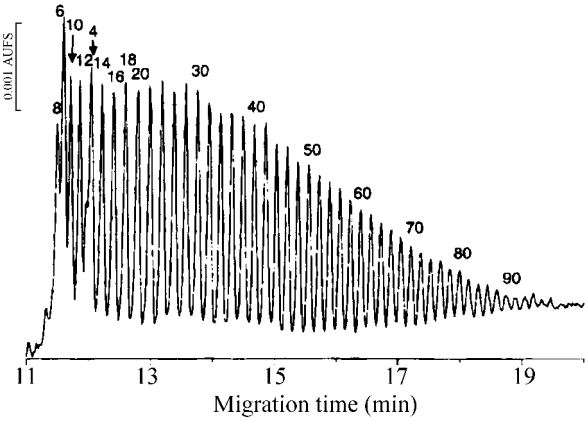

Figure 4. Analytical conditions: capillary, fused-silica capillary coated with (50% phenyl) methylpolysiloxane (57 cm × 100 μm i.d.; effective length, 27 cm); buffer, 0.1 M Tris–0.25 M borate (pH 8.5) containing PEG 70 000 at 10%; applied voltage, 10 kV (6.2 μA). The sample solution (1 mg/mL) was introduced for 10 s at 5 kV by the electrokinetic method. (Reprinted from *Anal. Chem.* 1999; **71**: 1592–1596. Copyright © 1999 American Chemical Society.)

acid oligosaccharides with up to 190 saccharide units are well separated by CE using a combination of a chemically modified capillary and in a 0.1 M Tri-0.25 M borate pH 8.5 buffer containing 10% polyethylene glycol (PEG) 70000, with detection at 200 nm (Figs 3 and 4; Kakehi *et al.*, 1999).

Heparin, low molecular weight heparin and heparan sulfate

The reversed polarity CE behavior of heparin has been optimized in a migration buffer of pH 6.0. A capillary coated with polyacrylamide was used to suppress the electroosmotic flow. Heparin gives rise to a broad peak showing negligible resolution of the components having different substitution patterns along the polymer chain. Cationic compounds bound to sulfo groups can help to reduce the peak broadening (Stefansson and Novotny, 1994a). The CE analysis of various synthetic heparins has been achieved in low pH phosphate buffer (pH 3.0; Damm *et al.*, 1992). Toida and coworkers (Toida and Linhardt, 1996) demonstrated the high sensitivity CE analysis of copper (II)–heparin, LMW heparin and heparin sulfate in a pH 3.0 buffer by reversed polarity. Heparin shows the highest sensitivity, followed by LMW heparin and heparan sulfate. Percentages of molar disaccharide compositions for unfractionated heparins from porcine, bovine intestinal mucosa and from bovine lung were determined by CE in a 60 mM formic acid buffer (pH 3.4; Ruiz-Calero *et al.*, 1998). In addition, LMW heparin from bovine and porcine intestinal mucosa was analyzed as well (Ruiz-Calero *et al.*, 1998).

Chondroitin sulfate and dermatan sulfate

Longer chain oligosaccharides derived from chondroitin sulfate have been separated in a capillary filled with polyacrylamide gel and these gels may prove effective in determining the degree of polymerization (Grimshaw, 1997). Analysis of dermatan sulfate has been performed using acidic phosphate buffer (pH 3.5) and reversed-polarity CE (Steffanson and Novotny, 1994b). Separation of chondroitin sulfate and dermatan sulfate was achieved by reversed-polarity CE with sensitive detection at 240 nm, based on the formation of a copper (II) complex in copper sulfate solution at a migration buffer of pH 3.0 (Toida and Linhardt, 1996).

GAG mixtures

CE can be applied to identify the different classes of GAGs. GAGs can be selectively removed from a biological sample by treatment with specific enzymes (Linhardt, 1994b) and then analysis of residual intact GAG. The structural differences between heparin and heparan sulfate in biological samples can be determined by analyzing the disaccharides obtained by their separate depolymerization with different heparinases (Rassi, 1999; Karamanos *et al.*, 1996). A comparative study of the CE migratory behavior of heparin and of dermatan sulfate has been described using an uncoated fused-silica capillary and phosphate buffer at pH 3.5 in reversed-polarity mode. The migration times for the two polymers were found to be very similar (Malsch and Harenberg, 1996).

CE ANALYSIS OF UNDERIVATIZED GAG-DERIVED OLIGOSACCHARIDES

Enzymatic depolymerization of GAGs

Currently the most fruitful way to obtain precise information on GAG fine chemical structure requires their depolymerization into oligosaccharide fragments, using specific polysaccharide lyases. These enzymes cleave specific glycosidic linkages between aminosugar and uronic acid residues present in GAGs and result in unsaturated oligosaccharide products that have UV absorbance at 232 nm (Linhardt *et al.*, 1986). CE analysis of the repeating disaccharide units permits the accurate determination of their sulfation patterns (Karamanos *et al.*, 1995, 1996). Uronic acid type, glucuronic acid or iduronic acid, can often be deduced by sensitivity to specific polysaccharide lyase (Desai *et al.*, 1993a,b; Linhardt, 1994b). Larger oligosaccharides prepared from GAGs can also be analyzed by CE (Pervin *et al.*, 1994; Desai *et al.*, 1993b). Such determination of the fine structure of oligosaccharides is useful for longer sequences with the same backbone repeating units.

The polysaccharide lyases are derived from a wide variety of pathogenic and nonpathogenic bacteria and fungi (Linhardt *et al.*, 1986; Southerland, 1995; Ernst *et al.*, 1995; Linhardt, 1994a,b). GAG-degrading enzymes are divided into two distinct classes: prokaryotic enzymes, which are lyases that depolymerize GAGs by an elimination mechanism (Linhardt *et al.*, 1996) and eukaryotic enzymes, which act by hydrolysis (Medeiros *et al.*, 1998). The lyase class of enzymes includes heparin lyases, chondroitin lyases and hyaluronate lyases (Linhardt *et al.*, 1986; Ernst *et al.*, 1995; Southerland, 1995).

Three different heparin lyases have been thoroughly investigated. Heparin lyase I acts on both heparin and heparan sulfate to produce the more highly sulfated disaccharides. Heparin lyase II has a very broad specificity, it is able to degrade heparin to a greater extent than heparin lyases I, as it can cleave disaccharide units containing two or three sulfate groups, and when used in combination with heparin lasese I and III can almost completely convert heparin and heparan sulfate to disaccharide (Desai *et al.*, 1993a,b). Heparin lyase III

acts only on heparan sulfate with a specificity affording the undersulfated disaccharides (Linhardt and Hileman, 1995).

Three classes of chondroitinase have been biochemically characterized based on their catalytic mechanism. Chondroitin lyase-ABC is a general purpose enzyme reacting with all members of the chondroitin sulfate group and also with hyaluronic acid. Chondroitin lyase-ABC cleaves the glycosidic linkages between glucuronic acid or iduronic acid and *N*-acetyl-galactosamine. This enzyme preparation is often a mixture of both an endolytic and exolytic enzyme (Hamai *et al.*, 1997). Chondroitin lyase-AC acts on chondroitin and dermatan sulfates, attacking only linkages containing glucuronic acid. Chondroitin lyase-AC can also act on hyaluronic acid. Chondroitin lyase-B cleaves aminosugars attached to iduronic acid (Jandik *et al.*, 1994).

Two distinct types of hyaluronate lyase are known, one being a hydrolase from animal orgin and the other an eliminase from bacterial sources. Hyaluronate lyase cleaves the linkage between *N*-acetyl-glucosamine and glucuronic acid, leaving oligomers with the *N*-acetyl-glucosamine as reducing terminus. The hyaluronate lyase from *Streptomyces hyalurolyticus* is specific for hyaluronic acid and does not act on the related GAGs, chondroitin sulfates (Linhardt *et al.*, 1986).

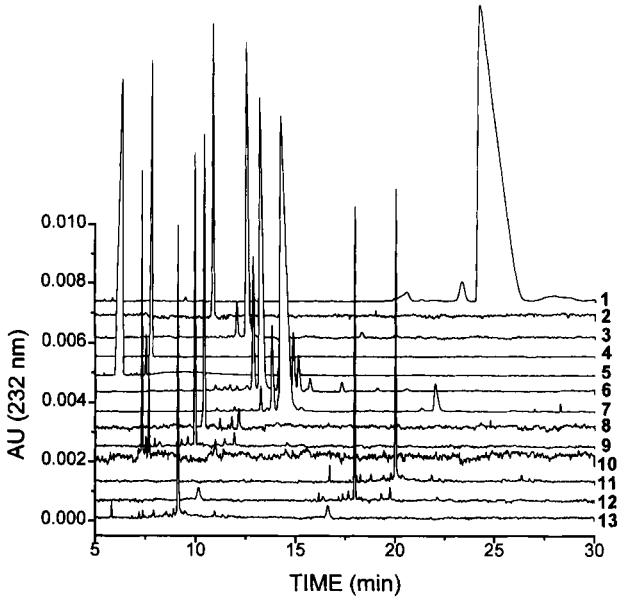

Figure 5. Reversed polarity capillary electrophoresis analysis of the heparan sulfate-derived oligosaccharides depolymerized by heparin lyase II or heparan lyase III. For the structure of the corresponding oligosaccharides see Hileman *et al.* (1997). (Reprinted from *Glycobiology* 1997; **7**: 231–239. Copyright © 1997 Oxford University Press.)

CE analysis of underivatized GAG-derived oligosaccharides

Hyaluronan oligosaccharides. Quantitative and qualitative determination of hyaluronic acid is easily achieved by CE. Hyaluronic acid is first degraded with hyaluronate lyase. The resulting hyaluronic acid-derived tetrasaccharide migrates as a sharp peak at a sodium phosphate/borate buffer (pH 9.0), with direct UV detection at low wavelengths (185–214 nm; Hayse *et al.*, 1997; Grimshaw *et al.*, 1996). Hyaluronic acid-derived oligosaccharides have also been separated in sodium phosphate/borate buffer in the presence of sodium dodecyl sulfate (Carney and Osborne, 1991).

Hyaluronic acid samples with different molecular weight were quantitatively determined by CE in a bare fused silica capillary using a slightly acidic buffer (pH 4.0) as the running electrolyte (Hayse *et al.*, 1997). Analysis of hyaluronic acid-derived disaccharides has also been successfully performed with an acidic phosphate buffer (pH 3.0; Karamanos *et al.*, 1995). Using reversed-polarity CE and detection at 232 nm, a high detection limit at the attomole level was obtained.

Hyaluronic acid-derived tetra-and hexasaccharides were well separated by CE based on borate buffer in the presence of an ion-pairing reagent, tetrabutylammonium phosphate (Payan *et al.*, 1998). The detection of hyaluronic acid depolymerization by CE has been adapted to determine hyaluronate lyase activity in bee and snake venom (Pattanaargson and Roboz, 1996).

Heparin and heparan sulfate oligosaccharides. The application of CE to total compositional analysis of heparin and LMW heparin samples has been studied. The heparin-derived disaccharide is separated by CE in an alkaline borate buffer pH 8.8 using normal polarity (Ampofo *et al.*, 1991). Under these conditions, the electroosmotic flow, not electrophoretic mobility, controls the separation of all the disaccharides. Isomeric species, however, migrate very closely to each other. Desai *et al.* improved the CE separation of heparin-derived oligosaccharides (Desai *et al.*, 1993b). Optimum resolution of 17 defined oligosaccharides was obtained with the buffer system composed of 10 mM sodium borate and 50 mM sodium dodecyl sulfate at pH 8.81 and at a constant voltage of 20 kV. The heparan sulfate-derived disaccharides can also be efficiently separated by an alkline borate buffer containing triethylamine and using normal polarity (Scapol *et al.*, 1996). Mixtures containing heparin and dermatan sulfate disaccharides can also be separated using fused-silica capillaries through the addition of triethylamine as electrolyte to a running buffer containing borate (Scapol *et al.*, 1996).

CE methods for the analysis of heparin and heparan sulfate-derived oligosaccharides have been developed using acidic buffer and direct UV detection. Heparin-derived disaccharide has been separated in a 20 mM

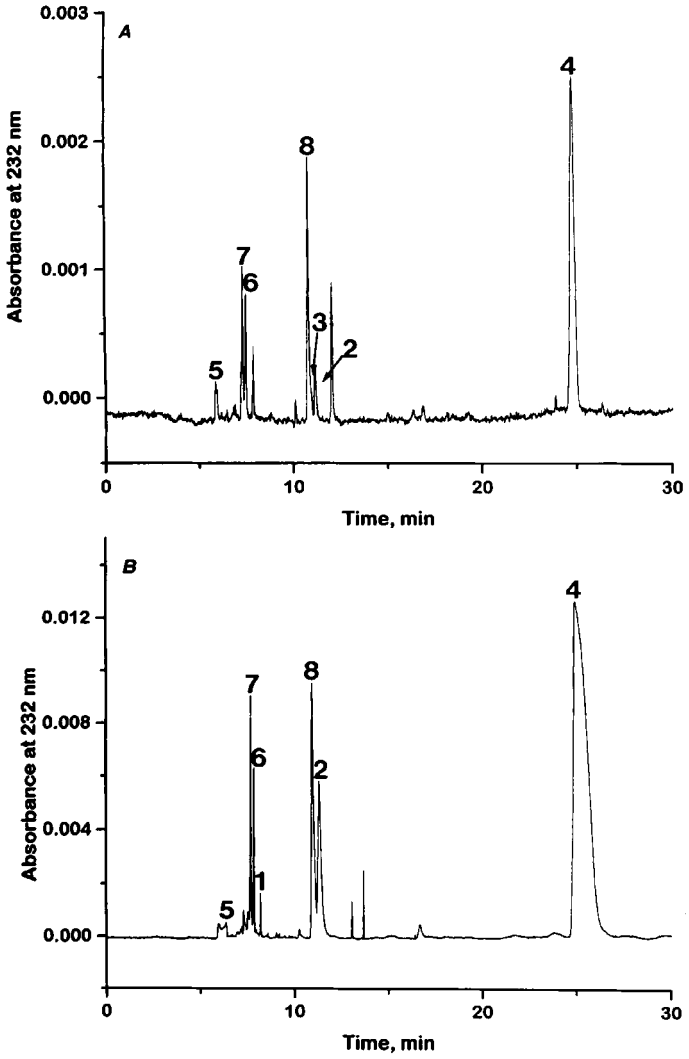

Figure 6. Capillary electropherograms of heparan sulfated-derived disaccharide treated with an equal-unit mixture of heparin lyases I, II and III. The CE system was operated in the reversed-polarity mode by applying the sample at the cathode and running using 20 mM phosphoric acid, pH 3.5, 15 kV with detection at 232 nm. (Reprinted from *Biochem. J.* 1997; **322**: 499–506. Copyright © 1997 Portland Press.)

phosphate buffer at pH 3.0 (Damm *et al.*, 1992). Under acidic conditions the electroosmotic flow is weak, relative to the electrophoretic mobility of negatively charged species. Disaccharides are eluted as sharper peaks than in the normal polarity mode and with baseline resolution. Pervin *et al.* compared the CE resolution of 13 heparin-derived oligoasaccharides of sizes ranging from disaccharide to tetradecasaccharide under either normal or reversed polarity (Pervin *et al.*, 1994b). A change between the low pH and the high pH migration buffers results in sample components being eluted in the reverse order. Under acidic conditions oligosaccharides are less well resolved under reverse polarity conditions at pH 3.8 than with normal polarity mode at pH 8.8 (Pervin *et al.*, 1994b). The resolution of reversed-polarity separation decreases with increased oligosaccharide size (Pervin *et al.*, 1994b). The eight heparin-derived disaccharides obtained by enzymatic depolymerization were separated by CE under a 60 mM formic acid buffer (pH 3.4), detection in the UV at 232 nm (Ruiz-Calero *et al.*, 1998). The purity of 13 homogenous heparan sulfate-derived oligosaccharides depolymerized by heparin lyase II or heparin lyase III was demonstrated by the presence of a single peak on reversed polarity CE (Fig. 5; Hileman *et al.*, 1997).

All known 12 unsaturated disaccharides derived from heparin and heparan sulfate can be separated in a single run of 15 min at 232 nm in a reversed polarity mode,

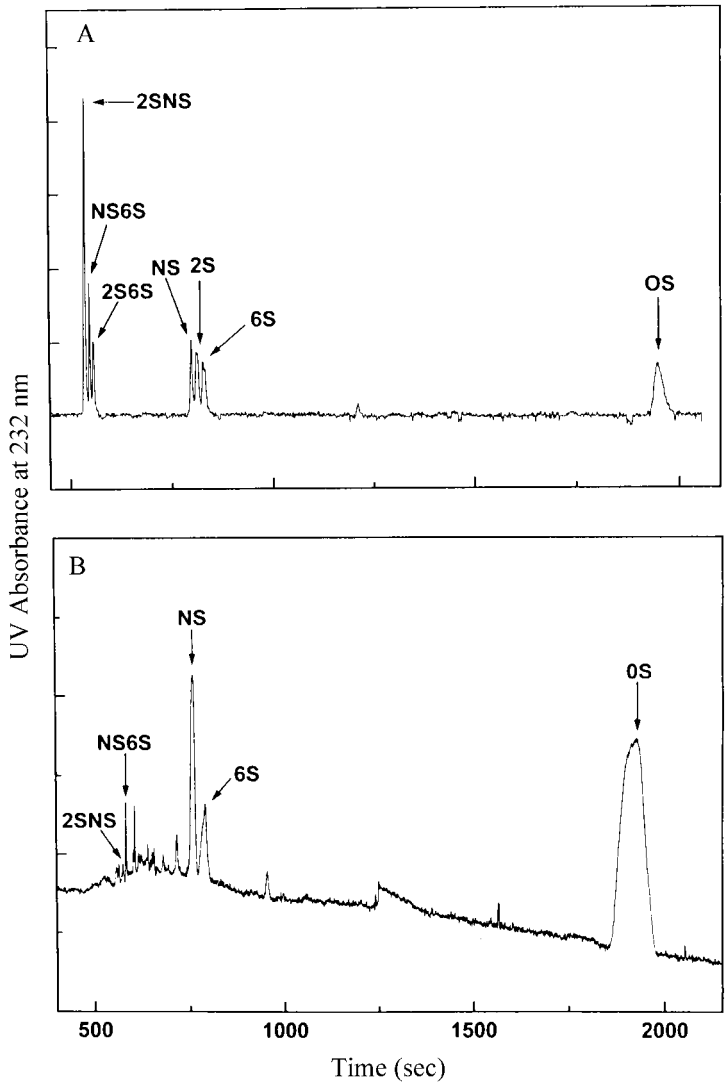

Figure 7. Capillary electropherogram of heparan sulfate GAG from purified proteoglycan used in the disaccharide compositional analysis. Panel (A) shows heparin and heparan sulfate disaccharide standards. Panel (B) shows the disaccharide composition of the heparan sulfate GAG from purified proteoglycan fraction after treatment with a mixture of equal amounts (on an activity basis) of heparin lyase I, II and III. The CE system was operated in the reversed polarity mode by applying the sample at the cathode and running with 20 mM phosphate, pH 3.5, 20 kV with detection at 232 nm. (Reprinted from *Biochem. J.* 1999; **344**: 723–730. Copyright © 1999 Portland Press.)

using 15 mM phosphate buffers (pH 2.5–5.0; Karamanos *et al.*, 1998; Lamari *et al.*, 1999). The heparin-derived oligosaccharides were analyzed by CE/MS. The separation electrolyte was made compatible with the requirements of a direct coupling using electrolytes made of ammonium acetate buffer, pH 3.5 and 9.2. Disaccharides that were not observed from the UV monitoring were identified (Duteil *et al.*, 1999). Toida and coworkers demonstrated the analysis of heparan sulfate-derived disaccharide by CE (Toida *et al.*, 1997a). Disaccharide analysis using CE relies on a charge-based separation that provides an accurate assessment of the sulfation of a GAG using disaccharide standards; nearly all peaks observed on reversed-polarity CE could be identified (Fig. 6). The disaccharide compositional analysis on the heparan sulfate chains from the three forms of the proteoglycan was determined by treatment with a mixture of heparin lyases followed by high-resolution CE. The results showed that they differed primarily by sulfo group content. The most highly sulfated proteoglycan isolated had a disaccharide composition similar to heparan sulfate found in brain tissue (Fig. 7, Park *et al.*, 1999).

Chondroitin sulfate and dermatan sulfate oligosaccharides. The application of CE to the analysis of disaccharides derived from chondroitin sulfate and dermatan sulfate was firstly reported by using a normal polarity CE in a basic borate buffer (Ampofo *et al.*, 1991; Carney and Osborne, 1991). The separation of 4S and 6S unsaturated disaccharides was further improved using a phosphate borate buffer (pH 9.0) containing sodium dodecyl sulfate (Scapol *et al.*, 1996; Ampofo *et al.*, 1991; Carney and Osborne, 1991). The separation of different types of mono- and disulfated $\Delta^{4,5}$-disaccharides may utilize two different operating systems: normal polarity for oversulfated species and reversed polarity for non- and monosulfated $\Delta^{4,5}$-disaccharides (Ampofo *et al.*, 1991; Carney and Osborne, 1991). The addition of triethylamine to an alkaline borate migration buffer (pH 8.8–10.40) also improves of the separation of chondroitin sulfate or dermatan sulfate disaccharides through an ion-pairing effect of the trimethylammonium ion (Scapol *et al.*, 1996). A sodium phosphate/borate buffer (pH 7.0) containing cetyltrimethylammonium bromide is very effective for separation of variously sulfated disaccharides (Michaelsen *et al.*, 1993). CE with appropriately chosen borate buffer and pH yielded baseline resolution of the 4S and 6S unsaturated disaccharides obtained through the depolymerization of chondroitin sulfates (Denuziere *et al.*, 1997). The addition of cinnamic acid as an internal standard improved the reproducibility of the determination of the disaccharides. Unsaturated disaccharides of chondroitin sulfates were separated and quantified by CE at a borate buffer modified by the ion-pairing reagent, tetrabutylammonium phosphate (Payan *et al.*, 1998).

All of the chondrotin sulfate-derived disaccharides can also be separated in one run, using fused-silica capillary, phosphate buffer (pH 3.0), under conditions of reversed polarity (Lillehoj and Alexander, 1992). Pervin *et al.* demonstrated that reversed-polarity CE completely resolves disaccharide mixtures from chondroitin sulfate and dermatan sulfate into all components using a single buffer, 20 mM phosphate-sodium phosphate at pH 3.48 (Pervin *et al.*, 1994b). The separation of eight chondroitin sulfate hexasaccharide fractions, isolated from commercial shark cartilage, was achieved by CE in 25 mM sodium phosphate buffer (pH 3.0) with detection at 185 nm. Dermatan sulfate-derived tetra-, hexa- and higher saccharides have been separated by CE (Karamanos *et al.*, 1995). Phosphate buffer (pH 3.0) under reversed-polarity conditions was employed for this separation. The results gave information on the iduronic and glucuronic acid repeats within the dermatan sulfate chain.

The purity of determan sulfate-derived oligosaccharides from porcine intestinal mucosal was confirmed by reversed-polarity CE (Yang *et al.*, 2000). Analysis of this mixture on CE demonstrated the presence of a complex mixture of oligosaccharide components grouped into clusters, and on reversed-polarity CE the most highly charged components elute first; strong-anion-exchange high-performance-liquid-chromatography (HPLC) shows the opposite separation pattern (Figure 8).

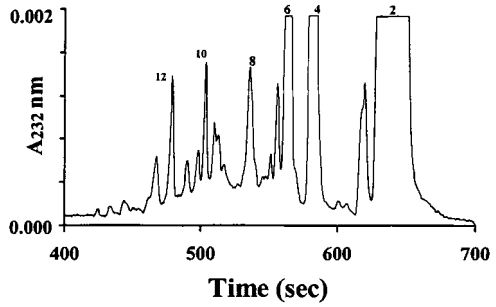

Figure 8. Capillary electrophoresis analysis of dermatan sulfate-derived oligosaccharides mixture depolymerized by chondroitinase ABC. The numbers indicate the degree of depolymerization. For the corresponding structures see Yang *et al.* (2000). (Reprinted from *Glycobiology* 2000; **10**: 1033–1040. Copyright © 2000 Oxford University Press.)

CE ANALYSIS OF DERIVATIZED GAGS AND GAG-DERIVED OLIGOSACCHARIDES

Derivatization methods

Derivatization not only provides a useful method for sensitive detection by fluorescence and/or UV, but also derivatization of carbohydrates can improve resolution of separation, through the introduction of charge and the alteration of analyte (Oefner and Chiesa, 1994; Hase, 1996).

The most frequently used method for the derivatization of carbohydrates is reductive amination. The reductive amination reaction takes place between the aldehyde group at the reducing end of sugar and amino group of labeling reagent employing sodium cyanoborohydride as a reductant. A number of labeling reagents have been used, including: 2-aminopyridine (2-AP; Forsberg *et al.*, 1991); 2-aminoacridone (AA or AMAC; Kitagawa *et al.*, 1995); 7-amino-1,3-naphthalenedisulphonic acid (AGA; Lee *et al.*, 1991); biotin aminopyridine (BAP; Toomre and Varki, 1994); *p*-aminobenzoic acid (Grill *et al.*, 1993); 2-aminobenzamide (Kinoshita *et al.*, 1999); 4-aminobenzonitrile (Schwaiger *et al.*, 1994); 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS; Stefansson and Novotny, 1994b; Klockow *et al.*, 1994; Chiesa and Horvath, 1993); 1-phenyl-3-methyl-5-pyrazolone (PMP; Honda *et al.*, 1992); 1-(4-methoxy)phenyl-3-methyl-5-pyrazolne (PMPMP; Kakehi and Honda, 1993); 6-aminoquinoline (6-AQ; Nashabeh and El-Rassi, 1992); 1-maltohepaosyl-1,5-diaminonaphthalene (Sudor and

Novotny, 1997) and 8-aminopyrene-1,3,6-trisulfonate (APTS; Cheng *et al.*, 1998).

The reductive amination of oligosaccharide with a sulfonated fluorescent label gives an oligosaccharide with a net negative charge identical to that of the label, even at low pH values. The greater the number of sugar residues in a particular oligosaccharide, the more vigorous are the conditions required to ensure complete derivatization. Reaction of a conjugated fluorescent amine with a sugar aldehyde group gives a Shiff-base that is stable at neutral pH values but which decomposes on exposure to acid (Kim *et al.*, 1995). Sodium cyanoborohydride reduces the Shiff-base forming an acid-stable secondary amine linkage between the sugar and the label. The migration of the labeled oligosaccharide is based on its molecular size (Park *et al.*, 1997). Amide bond formation between carboxylate groups and the amino group of a labeling reagent, using carbodiimide, also gives effective detection and separation (El Rassi *et al.*, 1997; Mechref *et al.*, 1995a,b; Honda *et al.*, 1992).

Depending on the chemical properities of the derivatives obtained, they can be detected by using UV, conventional fluoresence and/or laser-induced fluoresence. The sensitivity of UV detection is limited by the small path length. Conventional fluorescence, which is not path-length-dependent, can be used to increase the sensitivity. Laser-induced fluoresence has been also successfully used to enhance sensitivity even more (El Rassi *et al.*, 1997), however, laser-induced fluoresence detection is not yet widely used to analyze GAGs with CE. The constant excitation wavelength produced by laser limits the choice of chromophores.

CE for derivatized GAGs and GAG-derived oligosaccharides

The derivatization approach has been widely used in the analysis of GAGs and GAG-derived oligosaccharides. LMW heparin labeled with 1-maltoheptaosyl-1,5-diaminonaphthalene has been separated by CE. The end-label reagent also incorporates a fluorophoric group with desirable properties for laser-induced fluorescence detection (Sudor and Novetny, 1997). Fluorescence labeling of saturated and unsaturated saccharides with AA enabled the separation of chondroitin sulfate- and heparan sulfate-derived di- and oligosaccharides (Kitagawa *et al.*, 1995) and detection of separated species with laser-induced fluorescence detector.

All of the chondroitin and dermatan sulfate-derived, unsaturated disaccharides were derivatized with AA separated by CE using 15 mM orthophosphate buffer (pH 3.0), and detected using laser-induced fluorescence, enhancing analysis sensitivity by approximately 100-fold compared to UV detection at 231 nm (Lamari *et al.*, 1999; El-Rassi *et al.*, 1997). Labeling of dermatan sulfate-derived unsaturated disaccharides with AGA

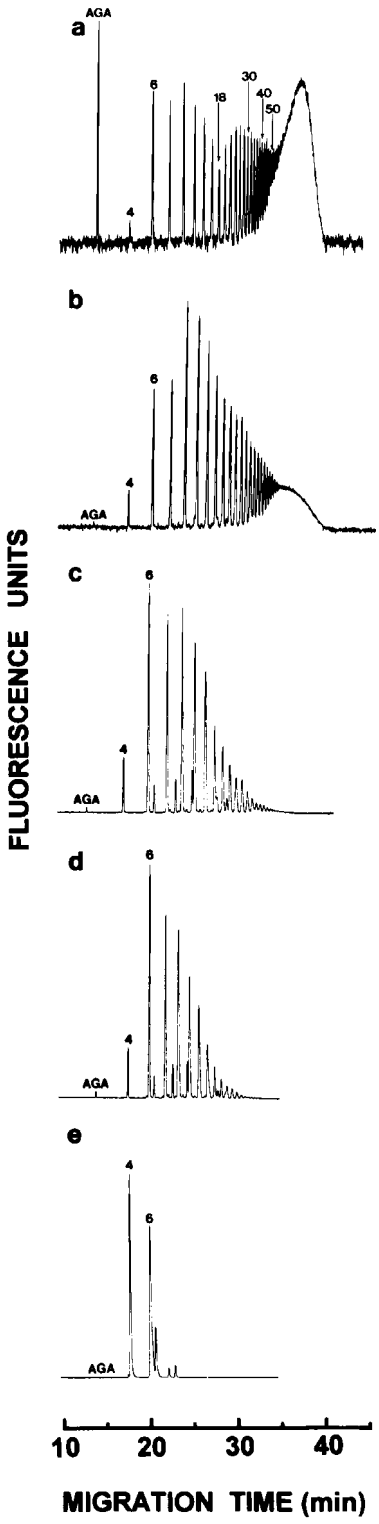

Figure 9. Capillary electropherograms of AGA labeled oligosaccharide mixtures prepared through controlled depolymerization of hyaluronic acid with hyaluronate lyase. Panels (a), (b), (c), (d) and (e) correspond to 16.9, 22.5, 43.7, 60.9 and 100% reaction completion. Each electropherogram contains AGA as an internal standard. The numbers indicate the degree of depolymerization (Park *et al.*, 1997). (Reprinted from *Biochim. Biophys. Acta* 1997; **1337**: 217–226. Copyright © 1997 Elsevier Science B.V.)

and CE analysis has been reported using laser-induced fluorescence detection (El-Rassi *et al.*, 1997). CE analysis of the chondroitin sulfate-derived unsaturated disaccharides labeled with AMAC was demonstrated by Nadanaka and Sugahara (1997). AGA-labeled oligosaccharides mixtures prepared through controlled depolymerization of hyaluronic acid with hyaluronate lyase were separated under reversed-polarity CE at pH 2.5 (Fig. 9). The peaks could be clearly assigned up to 50 saccharide units corresponding to a molecular weight of 9737. A plot of migration time as a function of the log of molecular mass demonstrates a nearly ideal linear relationship (Park *et al.*, 1997). Chondroitin sulfate-derived oligosaccharides have been labeled with PMP and analyzed in basic borate buffer pH 9.0 with detection at 214 nm, under normal polarity using flurorescence detection (Honda *et al.*, 1992). The increased sensitivity of this method permitted the disaccharide analysis of urinary glycosaminoglycans.

APPLICATIONS OF CE ANALYSIS OF GAGs

Quality control of GAGs in the pharmaceutical industry

The high sensitivity and accuracy of CE is an important method for quality control in biopharmaceutical manufacturing. The macromolecular configuration and rheological properties of hyaluronic acid have made this GAG interesting in ophthalmology and orthopedics, so that methods have been developed for separation and quantification of the components of commercial eye lotions and to maintain the depth of the anterior chamber in intraoccular lense insertion (Lamari *et al.*, 1998; Platzer *et al.*, 1999). Heparin finds considerable use in human medicine as an anticoagulant through its unique pentasaccharide sequence, which is involved in AT binding. The introduction of LMW heparins as new antithrombotic agents in the 1990s has made quality control of heparin raw material and the LMW heparin drug a priority in the pharmacueutical industry. Disaccharide analysis by CE is routinely performed on both heparin and LMW heparins (Pervin *et al.*, 1994b). Futhermore, it is also possible to estimate the variation of the number of pentasaccharide binding sites in different batches of LMW heparins (Desai *et al.*, 1995). The contamination of heparin and LMW heparin preparations with dermatan sulfate has been reported and is of some concern to the pharmaceutical industry and it is possible to use CE to assess such a contamination (Toida and Linhardt, 1996). In light of the appearance of 'mad cow disease' in humans, considerable effort is now directed towards developing CE methods for the characterization of the animal origin of heparin (Pervin *et al.*, 1994b; Scapol *et al.*, 1996). CE has been used in the analysis of chondroitin sulfates which are currently being used as nutriceuticals for relief of joint pain in the treatment of arthritis. CE analysis is important for quality control of chondroitin sulfate, currently being used in humans (Karamanos and Hjerpe, 1997).

GAG analysis for disease diagnosis

Analysis of heparan sulfate, heparin and heparin-derived oligosaccharides with AT binding sites is of importance in evaluating anticoagulation activity and coagulation disorders (Toida *et al.*, 1996; Gunnarsson *et al.*, 1997). The sulfation patterns of heparin and heparan sulfate can also be estimated by reversed-polarity CE, following treatment with heparin lyase I, II and III in combination (Karamanos *et al.*, 1996).

CE analysis of hyaluronic acid may provide useful information for diagnostic purposes, because of a multitude of biological processes of hyaluronic acid (Karamanos and Hjerpe, 1999). CE of disaccharides as a measure of hyaluronic acid content in pleural effusions has been a routine analysis in the diagnosis of human malignant mesothelioma (Nurminen *et al.*, 1994). Abnormal concentration of hyaluronic acid can also be demonstrated by CE in the exfoliation syndrome of the eye and in pathological conditions of the vitreous body (Grimshaw *et al.*, 1994).

The various sulfation patterns of chondroitin sulfate have been suggested as a marker to distinguish osteoarthritis from normal aging (Brown *et al.*, 1998). The development of atherosclerotic lesions in human aortas has also been linked to alterations in sulfation patterns of GAGs (Lamari *et al.*, 1999). Changes in the sulfation pattern for chondroitin sulfate have been characterized by reversed-polarity CE (Sharif *et al.*, 1996; Price *et al.*, 1996). CE can be used to estimate the variation in sulfation pattern of LMW dermatan sulfate, which is a key antithrombotic agent in blood vessels. Chondroitin sulfate concentration is significantly increased in tumors when compared to the amounts present in adjacent normal tissue. CE analysis of chondroitin sulfate in urine showed increased levels in patient with malignancies, indicating that the analysis might be used as a marker of malignancy (Dietrich *et al.*, 1993).

CE has also been adapted to the analysis of urinary GAGs (Honda *et al.*, 1992) and can thus be used to diagnose the mucopolysaccharidosis. Carney *et al.*, (1996) used CE to analyze and determine the fine structural functions of GAGs that are essential for antibody binding.

GAG analysis in glycobiology and carbohydrate biochemistry

CE has been used to analyze GAGs isolated from skin (Michaelsen *et al.*, 1993) and from the vitreous body

(Grimshaw *et al.*, 1994, 1996). The level of sensitivity makes CE particularly interesting when the preparation of large amounts is costly and/or tedious and it has been used to analyze GAGs in cultured cells (Karamanos *et al.*, 1995). A procedure to quantify hyaluronic acid in effusion from human malignant mesothelioma using CE is presented by Karamanos and Hjerpe (1997). CE is sensitive enough to determine hyaluronic acid in the synovial fluid, also where only small amounts of sample can be obtained in preosteoarthritic conditions, and to measure hyaluronic acid content and demonstrate the hyaluronic acid-protein/peptide interaction in biological samples (Grimshaw *et al.*, 1996; Price *et al.*, 1996). The interaction of heparin with AT and secretory leukocyte proteinase inhibitor has been studied by CE (Wu *et al.*, 1998). CE was used for a detailed characterization of the binding between heparin and a peptide isolated from the heparin-binding serum protein amyloid P component (SAP, Heegaard, 1998). It was also used to show that kinase insert domain containing receptor (KDR)-derived peptide interacts with a specific subset of polysaccharide chains contained in the unfractionated heparin (Dougher *et al.*, 1997). Microdetermination of heparin and other GAGs in plasma was achieved by CE (Toida *et al.*, 1997b).

Monitoring the chemical synthesis of GAG oligosaccharides

CE was first introduced as a routine method to follow development of synthetic chemical reactions by (Kerns *et al.* (1995). The high sensitivity of CE means that only a small amount of sample is required, making CE ideal for monitoring microscale reactions. The high resolution of CE permits the separation of complex mixtures that might be impossible to separate by TLC. The time required for CE sampling of the reaction is minimal and can be shortened further by reducing the capillary length or using a different buffer (Kuhr, 1993). Chemical modification of heparin-derived disaccharides and sulfated sucrose derivatives (important as anti-ulcer agents) can be conveniently followed using CE (Kerns *et al.*, 1995). Analysis times range from 10 to 60 min, and minimum sample preparation is required, permitting the sampling of an ongoing reaction. CE is useful for monitoring the synthetic sulfated bis-lactobionic acid amides, which are important heparin-like pharmaceuticals (Malá *et al.*, 1996) and isolate the desired species, which may partially decompose.

Other applications

The application of CE in enzymology involving GAGs and GAG-derived oligosaccharide substrates began as a simple outgrowth from oligosaccharide mapping experiments on GAGs. Heparin oligosaccharides were treated with various heparin lyases; their breakdown was followed by CE to study the specificity of each heparin lyase (Desai *et al.*, 1993a,b) or to confirm the structure of the oligoasaccharide starting material based on the known specificity of a heparin lyase. CE was also used to study the specificity of sulfoesterases acting on chondroitin sulfate and heparin-derived disaccharides (Pervin *et al.*, 1994a). The presence of small amounts of sulfoesterase contamination in commercial samples of polysaccharide lyases could also be assessed using CE.

An assessment of oligosaccharide purity may be determined using CE with detection by absorbance at 232 nm. Under both normal and reversed-polarity conditions, CE analysis showed a single symmetrical peak for each of the 14 oligosaccharides (Pervin *et al.*, 1995). The action pattern of *Streptomyces* hyaluronate lyase was analyzed by CE (Park *et al.*, 1997). The resulting electropherograms showed the content of each hyaluronic acid-derived oligosaccharide, having a degree of polymerization from 4 to 50, throughout the enzymatic reaction. Computer simulation studies have suggested that the hyaluronate lyase exhibits a random endolytic action pattern.

CONCLUDING REMARKS

The potential for the application of CE to the analysis of GAGs and GAG-derived oligosaccharides is excellent. The presence of a negatively charged group on these molecules ensures their migration under a variety of CE conditions. The power of the derivatization is that a simple, high-yield chemical step affords a GAGs derivative that is anionic and intensely fluorescent, facilitating both its separation and detection. The small sample requirements of CE are particularly suited for the analysis of valuable biological samples. CE is considered important for routine use in monitoring disease and biopharmaceuticals and the chemical synthesis of GAG oligosaccharides.

As science and technology progress towards more and more biological activity related to GAGs, the analysis and characterization of GAGs become more and more challenging. Refined structures of GAGs are key factors in determination the various biological functions. Alteration of these structures is associated with disease development and progress. To extend the analytical power of CE, new detection technology, new derivatization strategies and new CE-based separation methods which demonstrate increased speed, sensitivity and specificity must be developed. A processing challenge such as HPLC-CE or HPLC-CE-MS already points towards a developing trend.

Acknowledgements

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