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Review

Electrophoretic approaches to the analysis of complex polysaccharides

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Abstract

Complex polysaccharides, glycosaminoglycans (GAGs), are a class of ubiquitous macromolecules exhibiting a wide range of biological functions. They are widely distributed as sidechains of proteoglycans (PGs) in the extracellular matrix and at cellular level. The recent emergence of enhanced analytical tools for their study has triggered a virtual explosion in the field of glycomics. Analytical electrophoretic separation techniques, including agarose-gel, capillary electrophoresis (HPCE) and fluorophore-assisted carbohydrate electrophoresis (FACE), of GAGs and GAG-derived oligosaccharides have been employed for the structural analysis and quantification of hyaluronic acid (HA), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS), heparin (Hep) and acidic bacterial polysaccharides. Furthermore, recent developments in the electrophoretic separation and detection of unsaturated disaccharides and oligosaccharides derived from GAGs by enzymatic or chemical degradation have made it possible to examine alterations of GAGs with respect to their amounts and fine structural features in various pathological conditions, thus becoming applicable for diagnosis. In this paper, the electromigration procedures developed to analyze and characterize complex polysaccharides are reviewed. Moreover, a critical evaluation of the biological relevance of the results obtained by these electrophoresis approaches is presented.

Keywords: Glycosaminoglycans; Heparin; Dermatan sulfate; Chondroitin sulfate; K4 polysaccharide; K5 polysaccharide; Lipopolysaccharide; Electrophoresis

Contents

Abbreviations: 2-AMAC, 2-aminoacridone; CS, chondroitin sulfate; CSA, chondroitin-4-sulfate; CSC, chondroitin-6-sulfate; CZE, capillary zone electrophoresis; DS, dermatan sulfate; EM, electrophoretic mobility; EOF, electroosmotic flow; FACE, fluorophore-assisted carbohydrate electrophoresis; FMHep, fast-moving heparin; GAG(s), glycosaminoglycan(s); GalNAc, *N*-acetyl-galactosamine; GlcA, glucuronic acid; HA, hyaluronic acid; Hep, heparin; HS, heparan sulfate; HPCE, high-performance capillary electrophoresis; KS, keratan sulfate; LPS, lipopolysaccharide; MECC, micellar electrokinetic capillary chromatography; MEEKC, microemulsion electrokinetic capillary chromatography; PAGE, electrophoresis on polyacrylamide gel; PG(s), proteoglycan(s); SMHep, slow-moving heparin

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1. Introduction

Glycosaminoglycans (GAGs), hyaluronic acid (HA), keratan sulfate (KS), chondroitin sulfate (CS)/dermatan sulfate (DS), heparan sulfate (HS)/heparin (Hep), are linear, complex, polydisperse polysaccharides [1–6]. With the exception of KS, they consist of alternating copolymers of uronic acids and amino sugars, and their structures are commonly represented by typical disaccharide sequences [1,3,4]. Contrary to HA, KS, CS/DS and HS/Hep are sulfated heteropolysaccharides with different degrees of charge density due to sulfate groups in varying amounts and linked in different positions. They are very heterogeneous polysaccharides in terms of relative molecular mass, charge density, chemical properties, biological and pharmacological activities.

With the exception of HA, GAG chains are covalently attached at their reducing end through an *O*-glycosidic linkage to a serine residue or *N*-linked to asparagine (for KS) in a core protein; the ensuing macromolecules are termed proteoglycans (PGs) [7–10]. They are localized at cellular (intracellular granula and membrane-associated PGs) and extracellular levels where they play structural and regulating roles due to their interaction with several proteins [11].

Evidence obtained from recent glycobiology studies in the fields of biochemistry, cell and developmental biology suggests that PGs are not only structural components, but they participate in and regulate many cellular events and physiological processes, such as cell proliferation and differentiation, cell–cell and cell–matrix interactions [8,12–14]. The strong power of GAGs to be interactive at molecular and cellular level is due to their great structural diversity, as regards GAG type, size, composition and charge density, as well as the degree of substitution and domain arrangement. As a consequence, these heteropolyacids are a class of macromolecules of great importance in the fields of biochemistry, pathology and pharmacology.

Most GAGs are derived from animal sources by extraction and purification processes [1–6]. Recently, these natural substances have been chemically modified, and in addition synthetic analogues have been developed [15,16]. Currently, GAGs-based drugs represent natural or depolymerized HA, Hep, DS and CS, and mixtures of these polysaccharides. Hep derivatives devoid of anticoagulant action (with less *N*-sulfate and larger amounts of *N*-acetyl groups more closely resembling the structure of HS) are also utilized for pharmaceutical purposes. Source material, manufacturing processes, derivatization procedures, the presence of contaminants, and many other factors contribute to the overall biological and pharmacological actions of these agents.

HA is a linear polysaccharide composed of alternating residues of the monosaccharides D-glucuronic acid and *N*-acetyl-D-glucosamine linked by β(1 → 3) bonds in repeating units (Fig. 1). Disaccharides are linked to each other by β(1 → 4) bonds [17-18].

KS chains are known to be based upon a repeating *N*-acetyllactosamine sequence of – β(1-3)-[D-galactose-β(1 → 4)-*N*-acetyl-D-glucosamine]-β(1–3) – which is usually sulfated at C-6 of acetyl-glucosamine, and further sulfate groups may be present at C-6 of galactose [19] (Fig. 1). KS is a component of interstitial PGs, such as aggrecan and fibromodulin, involved in controlling elasticity and other rheological properties of the extracellular matrix, and in regulating the formation of the collagen fibrils network [20].

CSs are composed of alternate sequences of D-glucuronic acid and differently sulfated residues of *N*-acetyl-D-galactosamine linked by β(1 → 3) bonds. The regular disaccharide sequence of CSA, chondroitin-4-sulfate, is constituted by [(1 → 4)-*O*-(D-glucopyranosyluronic acid)–(1 → 3)-*O*-(2-*N*-acetamido-2-deoxy-D-galactopyranosyl-4-sulfate)]. CSC, chondroitin-6-sulfate, is mainly composed of a disaccharide unit [(1 → 4)-*O*-(D-glucopyranosyluronic acid)–(1 → 3)-*O*-(2-*N*-acetamido-2-deoxy-D-galactopyranosyl-6-sulfate)] (Fig. 1). Polysaccharide chains of DS (chondroitin sulfate B) consist of a prevailing disaccharide unit [(1 → 4)-*O*-(idopyranosyluronic acid)–(1 → 3)-*O*-(2-acetamido-2-deoxy-D-galactopyranosyl 4-sulfate)] (Fig. 1). Disaccharides with a different number and position of sulfate groups can be located, in various percentages, within the polysaccharide chains, such as the non-sulfated or disulfated disaccharide in which two sulfate groups are *O*-linked in position 2 of D-glucuronic acid and 6 of *N*-acetyl-D-galactosamine (disaccharide D) or in position 4 and 6 of *N*-acetyl-D-galactosamine (disaccharide E) (Fig. 1). These heterogeneous structures are responsible for the various and more specialized functions of these GAGs [21].

HS and Hep have a heterogeneous structure due to the presence of variously sulfated regions distributed along the chains. They are polysaccharides composed of alternate sequences of differently sulfated residues of uronic acid (D-glucuronic acid and L-iduronic acid) and *N*-acetyl-D-glucosamine or *N*-sulfo-D-glucosamine linked by α(1 → 4) bonds. Sulfate groups can be *O*-linked in position 2 of uronic acids, in position 6 of *N*-acetyl-D-glucosamine and in position 3 and 6 of *N*-sulfo-D-glucosamine [1,3,4] (Fig. 1). HS chains consist of a high percentage of glucuronic acid, *N*-acetyl-D-glucosamine and low-charged disaccharides (Fig. 1) whereas, on the contrary, Hep chains are formed by a high percentage of iduronic acid, *N*-sulfo-D-glucosamine and high-charged disaccharides. On the other hand, specific kinds of HS molecules are composed of sequences typical of Hep conveying Hep-like properties, such as anticoagulant capacity, to these polysaccharides.

Several analytical approaches (for reviews see [22–27]) have been developed to evaluate the species and quantity of various GAGs purified from biological samples. These are used to determine polysaccharides extracted from various organs, tissues and cells, and biological fluids, to identify modifications due to pathological conditions, and to measure quantitatively "pure" GAGs and their mixtures used as drugs. Moreover, analytical techniques are of paramount importance for evaluating the purity of a single GAG species used in therapy (Hep and DS as anticoagulant and antithrombotic drugs, CS as a condroprotective agent, and HA as an adjunct in eye surgery).

The usual methods are based on separation and quantitation of GAG species in mixtures by electrophoresis. In this review, the main electrophoretic systems to separate, analyze and quantify GAGs and derivatives are illustrated, including agarose-gel,

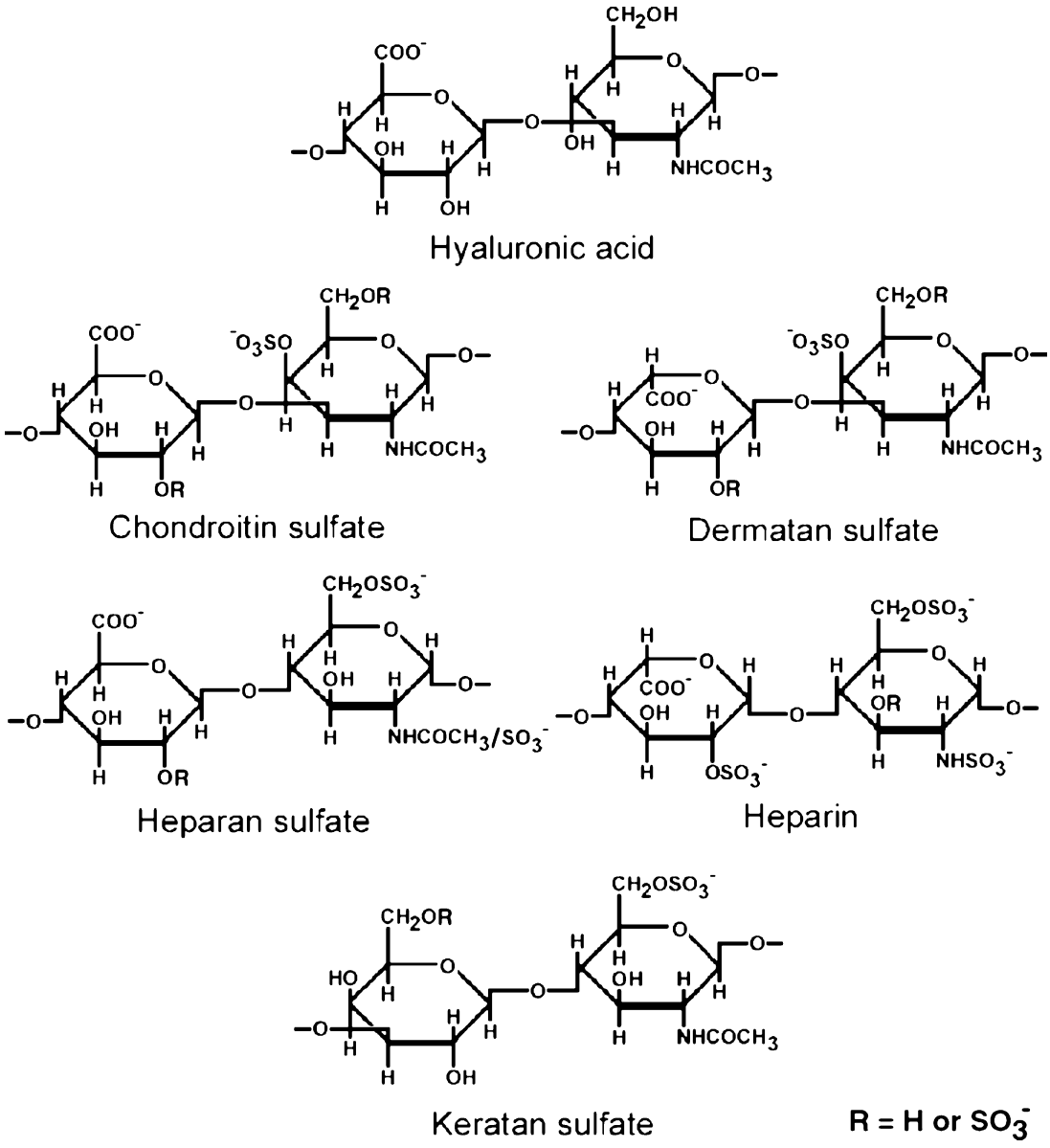

Fig. 1. Structures of disaccharides forming GAGs. Major modifications for each structure are illustrated (R = H or SO_3^-) but minor variations are possible.

capillary electrophoresis (HPCE) and fluorophore-assisted carbohydrate electrophoresis (FACE).

2. Electrophoresis on cellulose acetate

Electrophoretic separation of polysaccharides on cellulose acetate strips has been used for a long time. The use of barium acetate as a buffer facilitates the analytical separation of HA, DS, CS, and Hep [28]. Separation in hydrochloridic acid also represents a simple micromethod for GAG evaluation depending on their degree of sulfation [29]. Furthermore, a quantitative analysis of several GAGs was performed by separation of polysaccharides on cellulose acetate, alcian blue staining and densitometric scanning [30]. Several GAGs, HA, Hep, HS, KS, and isomeric CSs, CSA, DS, and CSC, can be separated by two-dimensional electrophoresis [31,32]. A rapid and sensitive method to separate HS, CSA, CSC, DS, HA, Hep, and KS is represented by a single monodimensional electrophoresis on cellulose acetate in a barium acetate buffer by using the differential sensitivity of polysaccharides to precipitation by ethanol [33]. KS, HS, DS, Hep, CSA, and HA were separated, presumably on the basis of their differential binding to the amino groups on ethylenediaminetetraacetic acid and lithium cations in the same electrophoretic solution. This method permitted the rapid separation and quantitative evaluation of these polysaccharides following a single electrophoretic procedure [34]. Using ^{103}ruthenium red, GAGs can be detected on cellulose acetate electrophoretograms at much greater sensitivity, at the nanogram level, in comparison with other methods [35].

However, electrophoresis on cellulose acetate entails various progressive electrophoretic steps in different buffer/ethanol solutions [33] otherwise it does not permit the separation of FMHep, possessing low molecular mass and charge density, and SMHep, having higher molecular mass and charge density, components of Hep [29,36].

3. Electrophoresis on nitrocellulose membrane

A rapid and simple electrophoretic separation on nitrocellulose membranes by a single run of GAGs, permits the qualitative and quantitative analysis of SMHep and FMHep, DS, HA and CS [37]. The detection limit for FMHep, DS and CS was about 0.2–0.3 μg, whereas that for SMHep was 0.1 μg by using azur-A.

4. Agarose-gel electrophoresis

Agarose-gel electrophoretic separation of polysaccharides was investigated for the first time by Dietrich et al. [38,39]. In a barbital buffer the polysaccharides were fractionated from each other as a function of their net charge, whereas in a diamine buffer the fractionation was achieved according to the degree to which they are bound to the diamine. A combination of barbital and diaminopropane buffers in two-dimensional electrophoresis for the identification of complex polysaccharides was developed. Since its first application, many modifications and applications of this electrophoretic technique have been performed and at the moment this is one of the most qualitative and quantitative analytical approaches for the evaluation of GAGs. In fact, agarose-gel electrophoresis has been applied for various purposes, such as to separate GAGs extracted from tissues, organs, biological fluids of invertebrates and vertebrates, to characterize radiolabelled sulfated polysaccharides from cells, to evaluate polyanions used as drugs both qualitatively and quantitatively [40,41], to control a purification step of a single GAG species [41], to evaluate polysaccharide–protein interactions [42]. Agarose-gel electrophoresis is a useful technique to analyze GAGs in mixtures. This approach permits the separation of the main acidic polysaccharides, such as Hep with its two components, the more sulfated and with higher molecular mass SMHep and the less sulfated with lower molecular mass FMHep, HS, DS and CS (Fig. 2). Furthermore, microanalyses are of paramount importance to evaluate the structure and properties of GAGs modified by chemical or enzymatic procedures, such as depolymerized, desulfated and deacetylated derivatives [43], and in particular for Hep composed of two species easily detachable by agarose-gel electrophoresis (Fig. 3A). Besides, densitometric analysis of bands enables us to obtain quantitative evaluation of single polysaccharide species in mixtures (see for example, Fig. 3B). Thanks to these functions, agarose-gel electrophoresis gives us more detailed information on the complex polysaccharides when used in combination with other analytical approaches, such as polyacrylamide-gel electrophoresis for the analysis of GAG oligosaccharides [44,45] and also of molecular size distribution [46,47] for single species of polysaccharides (see below), dot-blot assay

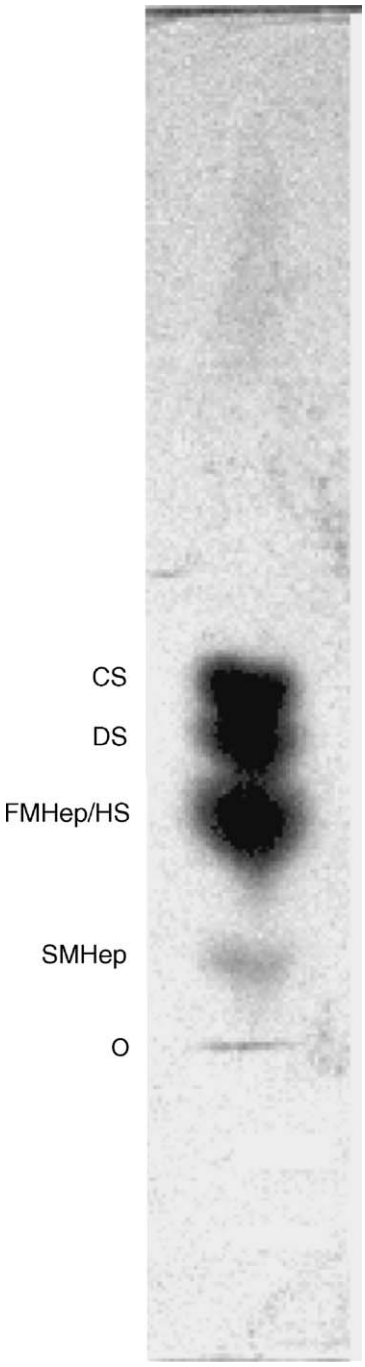

Fig. 2. Agarose-gel electrophoresis stained with toluidine blue of sulfated GAGs. FMHep and HS have the same migration properties under the adopted experimental conditions. o: origin.

for quantitative purposes [48], high-performance size-exclusion chromatography for the determination of polyanion molecular mass and polydispersity [49], and capillary electrophoresis [26] (see below).

Agarose-gel electrophoresis has also been applied to the identification of Hep samples having different molecular masses, from 11,600 to 1600 (Fig. 4), in particular to determine the relative percentage of SMHep and FMHep [50].

A procedure to analyze for purity, unsaturated disaccharides and molecular mass, sulfated GAGs in mixtures has been devel-

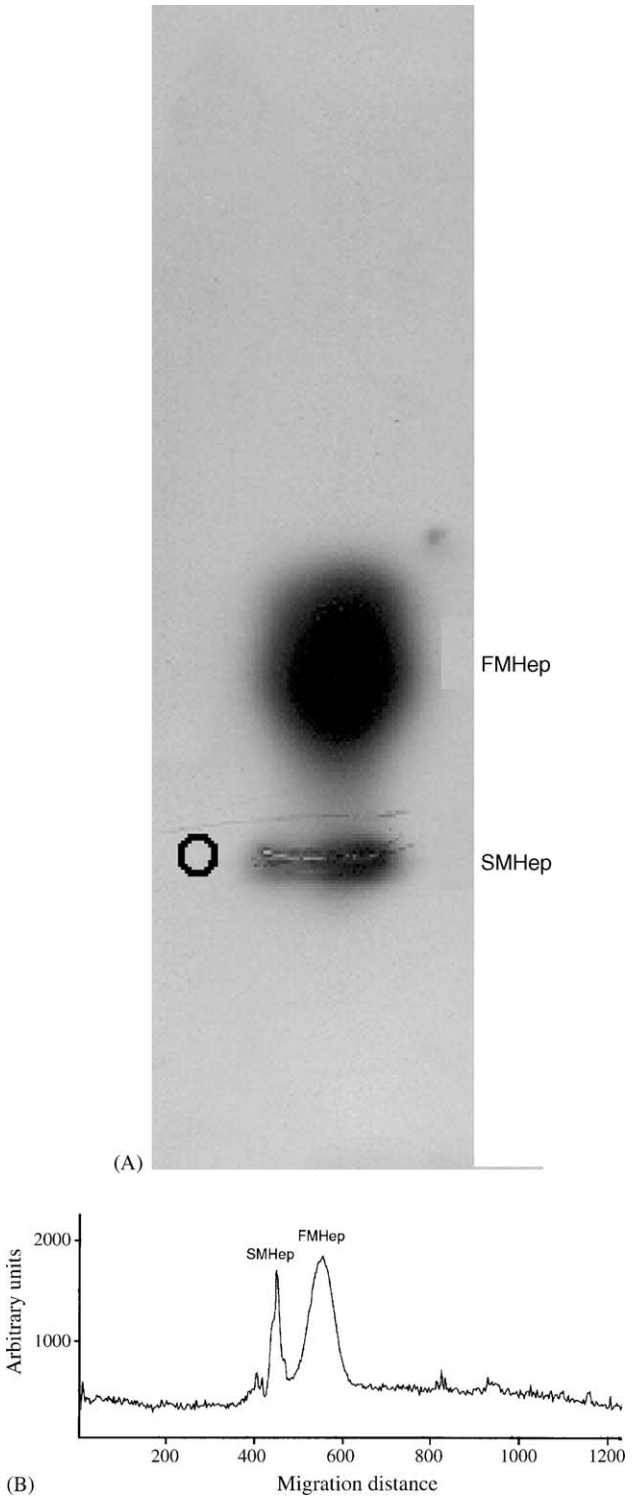

Fig. 3. (A) Agarose-gel electrophoresis of a heparin sample purified from *Callista chione* and (B) the densitometric scanning performed by a densitometer. o: origin (modified from [72]).

oped by recovering complex polysaccharides directly from gel matrix as single species [51]. Sulfated GAGs, in particular Hep with its two components, SMHep and FMHep, HS, DS, and CS, were separated to microgram level by conventional agarose-gel electrophoresis. After their separation, they were fixed in the agarose-gel matrix by precipitation in a cetyltrimethylammonium bromide solution, making them visible on a dark background. After recovery of gel containing the fixed bands, high temperatures (90 °C for 15 min) were necessary to dissolve the gel matrix, and a solution of NaCl (3 M) was used to release sulfated polysaccharides from the complex with cetyltrimethylammonium. After precipitation of GAGs in the presence of ethanol, the recovery of SMHep, FMHep, HS, DS and CS was obtained from 1 to 10 μg, with a percentage greater than 45% and a purity above 90%. Sulfated GAGs in mixtures recovered from gel matrix as single species were evaluated for purity, and characterized for unsaturated disaccharides after treatment with bacterial lyases (heparinases for Hep and HS samples, and chondroitinases for DS and CS) and molecular mass.

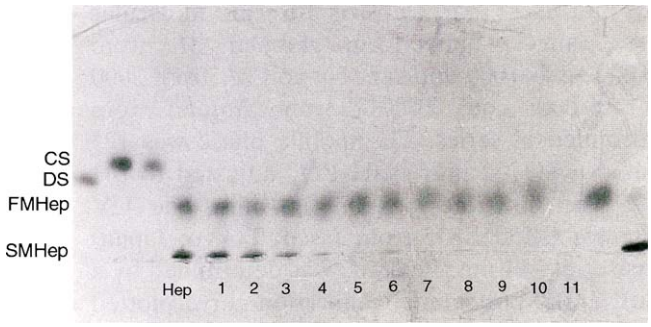

Fig. 4. Agarose-gel electrophoresis of standard GAGs (CS, DS, FMHep and SMHep) several Hep samples having different molecular masses, from 11,600 to 1620. (1) 11,660, (2) 7560, (3) 6300, (4) 5430, (5) 4560, (6) 3800, (7) 3640, (8) 3360, (9) 3030, (10) 2820, (11) 1620 (undetectable due to the very low molecular mass) (modified from [50]).

A method for blotting and immobilizing several nonsulfated and sulfated complex polysaccharides on membranes after their separation by conventional agarose-gel electrophoresis has been described [52]. Nitrocellulose membranes were derivatized with the cationic detergent cetylpyridinium chloride and mixtures of GAGs were capillary blotted after their separation in agarose-gel electrophoresis in barium acetate/1,2-diaminopropane (Fig. 5). Single purified species of variously sulfated polysaccharides were transferred onto the derivatized membranes after electrophoresis with an efficiency of 100% and stained with alcian blue (irreversible staining) and toluidine blue (reversible staining) permitting about 0.1 μg threshold of detection. Nonsulfated polyanions, HA, a fructose-containing polysaccharide with a chondroitin backbone purified from *Escherichia coli* U1-41 (polysaccharide K4), and its defructosylated product (K4d), were also electrophoretically separated and transferred onto membranes. The detection limit for desulfated GAGs was about of 0.1–0.5 μg after irreversible or reversible staining. Furthermore, the membrane stained with toluidine blue (reversible staining) was destained and the same lanes used for immunological detection [53]. Reversible staining was also applied to recover single species of polysaccharides for further biochemical characterization after electrophoretic separation of mixtures of GAGs and their transfer onto membranes.

The most common methods for the visualization of sulfated GAGs after their separation by electrophoresis are based on the metachromatic activity of polysaccharides and their com-

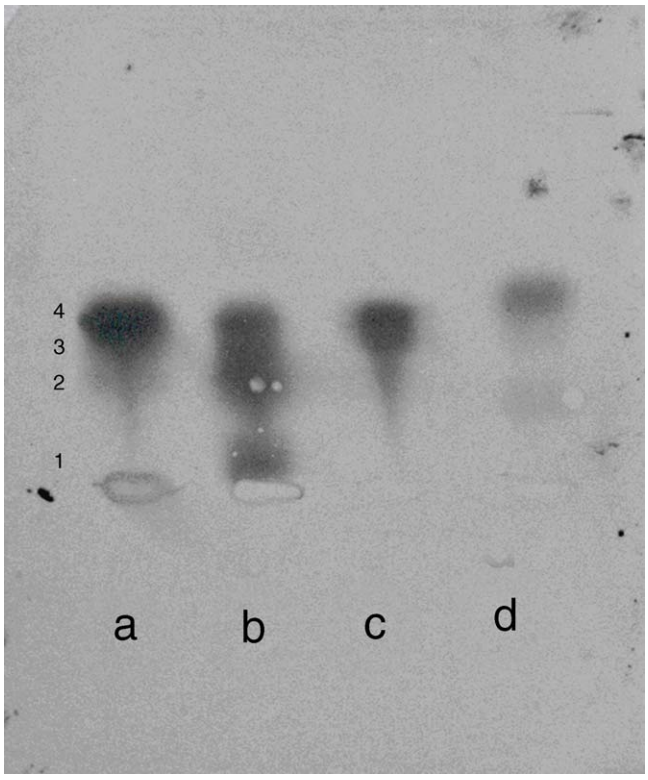

Fig. 5. GAGs extracted from several tissues and fluids (a) human aorta, (b) bovine lung, (c) bovine aorta, and (d) human urine, separated by agarose-gel electrophoresis (5 μg of purified GAGs) and blotted onto CPC-treated NC membrane. The membrane was stained with alcian blue. (1) SMHep, (2) FMHep or HS, (3) DS and (4) CS (reprinted with permission from [52]).

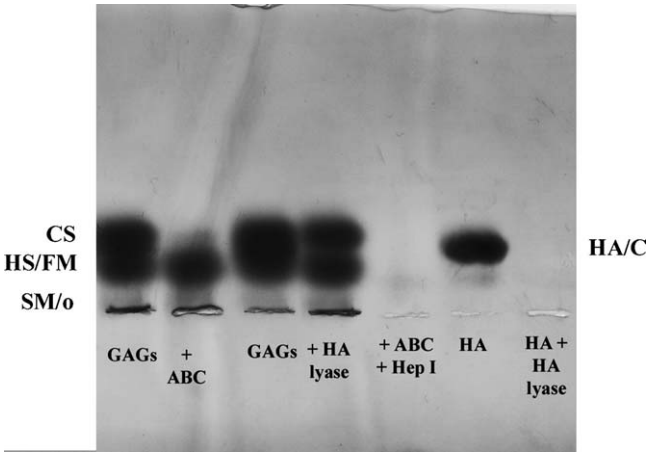

Fig. 7. Agarose-gel electrophoresis stained with toluidine blue and Stains-All of *Anodonta anodonta* GAGs, untreated and treated with various enzymes. GAGs: purified polysaccharides untreated; +ABC: extract treated with chondroitinase ABC; +HA lyase: extract treated with hyaluronidase SD from *Streptococcus dysgalactiae*; +ABC +Hep I: extract treated with chondroitinase ABC and heparin lyase I; HA: HA from rooster comb; HA + HA lyase: HA from rooster comb treated with hyaluronidase SD from *Streptococcus dysgalactiae*. o: origin (reprinted with permission from [62]).

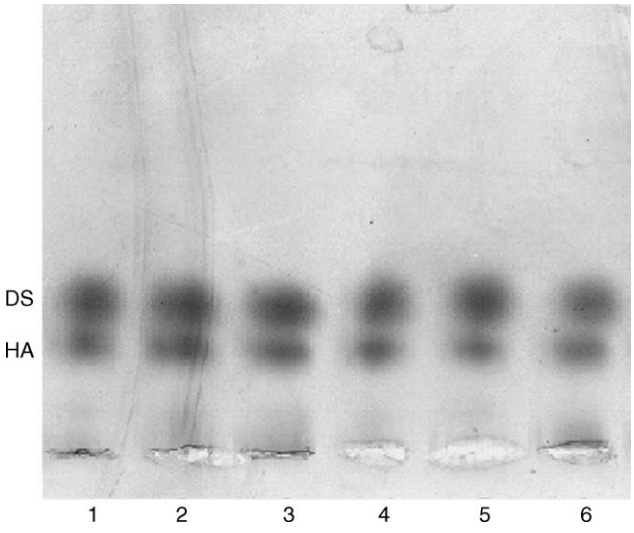

Fig. 6. Discontinuous agarose-gel electrophoresis in 0.05 M HCl/0.04 M barium acetate of GAGs extracted and purified from the skin of several rats (from 1 to 6) (reprinted with permission from [60]).

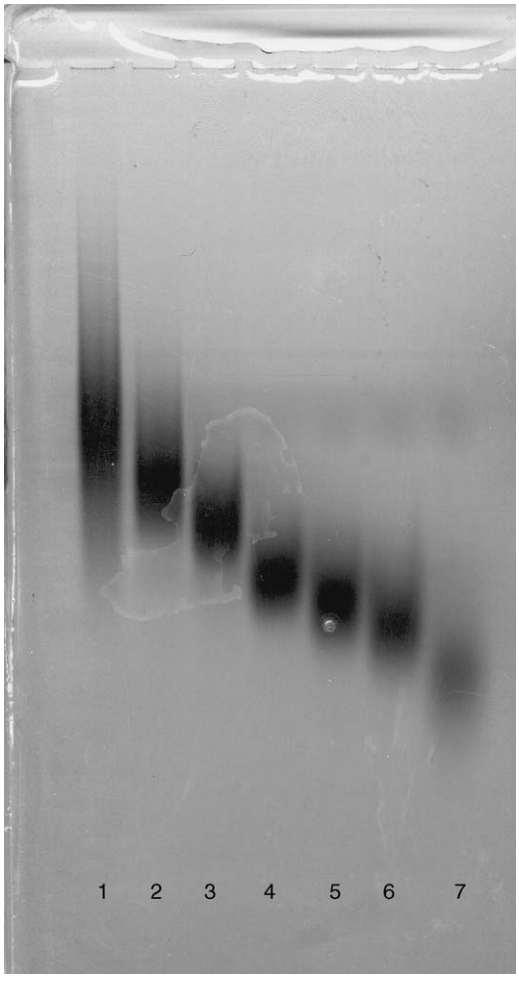

Fig. 8. PAGE separation of different molecular mass Hep samples. (1) 13,500, (2) 7560, (3) 6300, (4) 4560, (5) 3640, (6) 2820, (7) 1620.

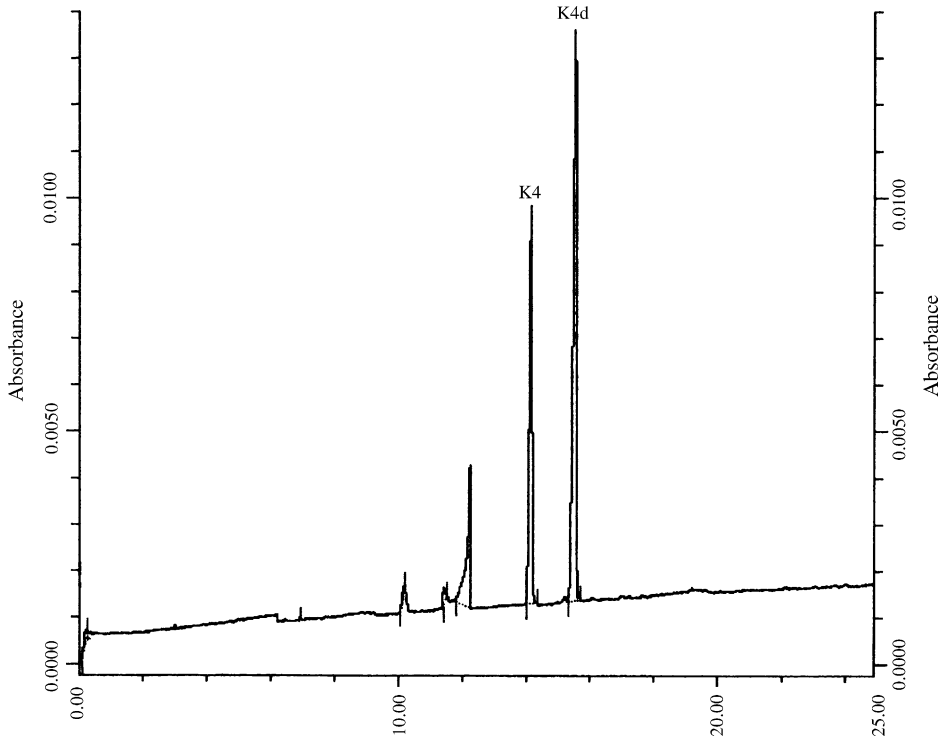

Fig. 9. HPCE electrophoregrams of unsaturated disaccharide ΔHexAFrc-GalNAc forming the polysaccharide K4 (K4) and of the ΔHexA-GalNAc of defructosylated K4 product (K4d) (reprinted with permission from [77]).

plexation with cationic dyes such as azure A, toluidine blue, alcian blue, and methylene blue. Toluidine blue is currently used in agarose-gel electrophoresis. The detection limit for the determination of GAGs with this method is from >0.1 to 1 μg (Table 1). Combined azure A/ammoniacal silver was also applied to agarose-gel electrophoresis to increase the sensitivity by about 200 times [54,55] (Table 1). However, this method entails several steps to fix and prestain the gel, followed by destaining, air-drying, treatment with ammoniacal silver solution, and stopping of the reaction. A new visualization technique using toluidine blue/Stains-All for GAGs separated on agarose-gel electrophoresis was established and it has been shown to be a valuable method for assaying several sulfated GAGs with a detection limit at submicrogram-level, as little as 10 ng of a single species, greater than about 10 times that of conventional procedures [56]. Furthermore, this technique was able to detect nonsulfated polysaccharides such as HA. Due to these capacities, agarose-gel electrophoresis stained with combined toluidine blue/Stains-All was successfully applied to the separation and detection of lipopolysaccharide samples from several *E. coli* serotypes [57], of the polysaccharides K4 [58] and K5 [59] from the uropathogenic *E. coli* K4 or K5 bacteria during their extraction and purification processes, and of the HA in combination with DS (Fig. 6) [60].

Agarose-gel electrophoresis separation of polysaccharides is a powerful analytical tool for the characterization of complex GAGs mixtures when associated with treatment with specific enzymes or chemical reactions, such as the degradation of Hep/HS with nitrous acid [61]. Fig. 7 shows as an example the agarose-gel electrophoresis stained with toluidine blue and Stains-All of *Anodonta anodonta* GAGs, both untreated and treated with various enzymes [62]. To identify the species of polysaccharides, the GAGs extract was subjected to treatment with specific enzymes, i.e. chondroitin ABC lyase, chondroitinase B, heparin lyase I, hyaluronate lyase from *Streptomyces hyalurolyticus* or hyaluronidase SD from *Streptococcus dysgalactiae*, and agarose-gel electrophoresis.

An improved technique for the agarose-gel electrophoresis separation of PGs has been developed by using a discontinuous buffer system that allows stacking of the sample [63]. Several PG fractions were used to establish the relationship between size and mobility in agarose-gels of different concentrations. By using this analytical approach, PGs of different sizes from various tissues and biological fluids were separated.

5. Electrophoresis on polyacrylamide gel (PAGE)

PAGE is a powerful tool to characterize the properties and structures of GAGs. For the first time, Hilborn and Anastassiadis [64] reported the possibility to determine the molecular weight of GAGs by PAGE, and Hsu et al. [65] their molecular size distribution. Cowman et al. [66] used PAGE to evaluate GAGs oligosaccharides differing in chain length by one disaccharide unit. Furthermore, combined alcian blue and silver staining of GAGs in PAGE permits detection below 50 ng per band [67]. Several Hep-derived oligosaccharides with different charge densities and degrees of polymerization were characterized by PAGE [68]. Al-Hakim and Linhardt [69] also reported

Table 1
Electrophoresis techniques and general conditions for the determination of polysaccharides and their derivatives

Electrophoretic technique	Limit of detection	GAGs detected	Procedure	Application	Reference(s)
Cellulose acetate	0.001–1 μg (alcian blue)	HA, HS, CSA, CSC, DS, KS, Hep	Discontinuous separation by using increasing % of ethanol	Evaluation of GAGs in mixtures and purity of single GAGs	[33]
Nitrocellulose	0.1–0.3 μg (azur-A)	HA, CS, DS, SMHep, HS/FMHep	Continuous separation	Evaluation of GAGs in mixtures and purity of single GAGs	[37]
Agarose-gel	>0.1 μg (toluidine blue)	CS, DS, SMHep, HS/FMHep	Discontinuous separation by using two different buffers	Evaluation of GAGs in mixtures and purity of single GAGs	[38,39]
Agarose-gel	1 μg (precipitation in cetyltrimethylammonium bromide)	CS, DS, SMHep, HS/FMHep	GAGs fixed in the agarose-gel and recovered	Evaluation of the structure and properties of GAGs	[51]
Agarose-gel	0.1 μg (alcian blue/toluidine blue)	Nonsulfated GAGs, CS, DS, SMHep, HS/FMHep	Blotting GAGs on membranes after electrophoretic separation and immunodetection by antobodies	Evaluation of the nature of GAGs	[52,53]
Agarose-gel	Approximately 0.001 μg (azur-A/ammoniacal silver)	HA, CS, DS, KS, HS, Hep	Sequential staining by means azur-A and ammoniacal silver	Evaluation of GAGs in mixtures and purity of single GAGs	[54,55]
Agarose-gel	0.01 μg (toluidine blue/Stains-All)	Nonsulfated GAGs, CS, DS, SMHep, HS/FMHep	Sequential staining by means toluidine blue and Stains-All	Evaluation of GAGs in mixtures and purity of single GAGs. Determination of the nature of GAGs	[56,62]
PAGE	0.05 μg (alcian blue/ammoniacal silver)	HA, CS, DS, HS, Hep	Discontinuous or continuous separation of single GAGs	Evaluation of the molecular mass and distribution of single GAGs	[67–70]
PAGE	0.001 μg (azur-A/ammoniacal silver)	Oligosaccharides from HA, CS, DS, HS, Hep	Discontinuous separation of GAG oligosaccharides	Evaluation of the structure and properties of GAGs	[73,74]
HPCE	0.03 μg (detection at 230 nm)	Nonsulfated GAGs, LPS	Electrokinetic chromatography with sodium dodecyl sulfate	Quantitative evaluation and determination of some properties of nonsulfated GAGs	[23,57–59]
HPCE	Depends on the technique	GAGs disaccharides and oligosaccharides	Depends on the technique	Qualitative evaluation and determination of the structure and properties of GAGs	([23–26] for review, [77])
FACE	Approximately 0.005 μg	Disaccharides and oligosaccharides from HA, CS, DS, KS, HS, Hep and bacterial polysaccharides	Separation of labeled derivatives based on a PAGE	Determination of the fine structure and properties of GAGs	[86,87]

the possibility to isolate and recover acidic oligosaccharides from PAGE by semi-dry electrotransfer. Gradient PAGE has also been applied to the determination of molecular weights of Hep preparations and low-molecular-weight Hep derivatives [70]. Linear PAGE in a barbital buffer (Fig. 8) has also been utilized for the determination of the molecular mass of Hep samples extracted and purified from several molluscs [71,72]. Actually, PAGE is a powerful technique for a rapid sequencing of HS and Hep saccharides [73] and for the characterization and quantitation of HA and CS/DS saccharides [74].

Electrophoresis on large-pore composite polyacrylamide–agarose-gel is usually applied to the separation and characterization of high molecular weight polymers such as PGs [75,76].

6. High-performance capillary electrophoresis (HPCE)

The commonest and simplest mode of HPCE is capillary zone electrophoresis (CZE) (for reviews see [23–26]). Upon voltage application, the negatively charged wall of the uncoated fused-silica capillary causes electroosmotic flow (EOF) of buffer species, which conveys all analytes towards the negative electrode (cathode). The separation of solutes is accomplished by the vector sum of EOF and their electrophoretic mobility (EM), depending on the charge to mass ratio of the molecules. In the case of GAGs-derived saccharides, which are inherently negatively charged, EOF and EM counteract each other, and the apparent migration depends on the net difference between these two driving forces. At acidic pH, EOF is so low that it cannot overwhelm the EM of anions. In this case, the polarity is reversed.

Micellar electrokinetic capillary chromatography (MECC) mimics reversed-phase HPLC conditions, since analytes interact with micelles in the operating buffer, which is also known as the pseudostationary phase. MECC can easily be used for separation of both charged and neutral solutes bearing either hydrophobic or hydrophilic characteristics. In GAGs analysis, the benefits of MECC have been exploited by the addition of detergents and other additives, such as sodium dodecyl sulfate and cetyltrimethylammonium bromide in the alkaline borate buffers commonly used [24–26].

Microemulsion electrokinetic capillary chromatography (MEEKC) is another recently introduced HPCE operation mode in which neutral and ionized species can be resolved according

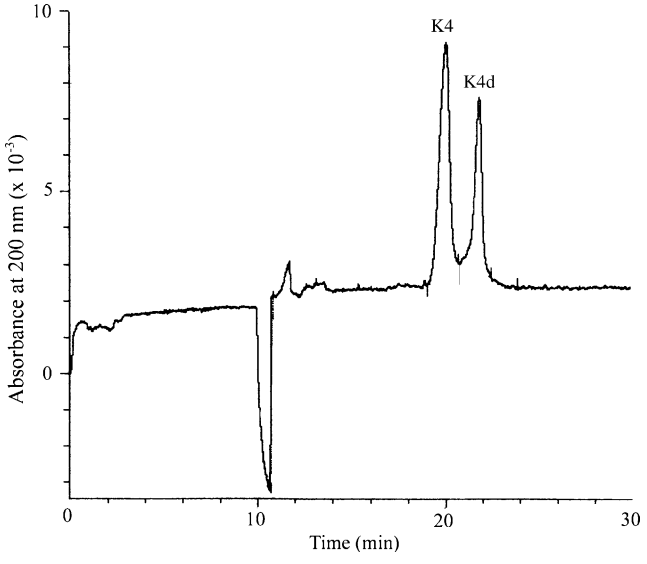

Fig. 10. HPCE electrophoregrams of the polysaccharide K4 (K4) and of the defructosylated K4 polysaccharide (K4d) (reprinted with permission from [58]).

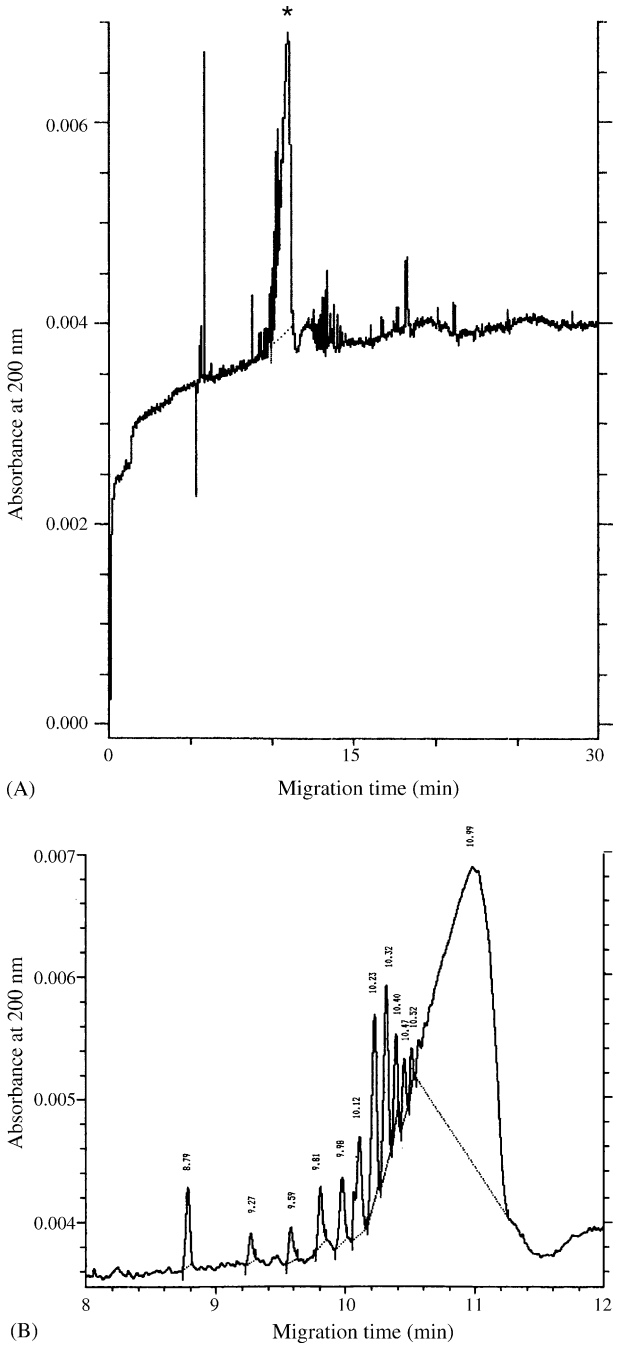

Fig. 11. (A) HPCE electrophoregrams of the purified *E. coli* K5 polysaccharide. The asterisk indicates the K5 species at a migration time of approximately 11.00 min. (B) HPCE electrophoregram of the K5 polymer as in (A) expanded in the region from 8.00 to 12.00 min. The K5 polysaccharide species with a migration time of 8.79 min corresponds to a decasaccharide (reprinted with permission from [59]).

to their partitioning into oil droplets moving in the operating buffer.

By and large, CZE analysis of different types of variously sulfated unsaturated-disaccharides involves two operating systems: one of reversed polarity at low pH and another of normal polarity at high pH. Analysis in acidic phosphate buffers at low pH and reversed polarity provides a rapid resolution of all variously sulfated GAG-derived disaccharides [24–26]. The only problem that has been observed in this system is the lack of reproducibility of the migration times of nonsulfated disaccharides, which may be attributed to the stability of the capillary used. The real benefits of HPCE are demonstrated by both the ease and speed of separation, the trace amounts of samples required, and the high sensitivity.

A rapid, highly sensitive and reproducible HPCE method (electrokinetic chromatography with sodium dodecyl sulfate) has been developed in our laboratory for the determination of disaccharides present in the polysaccharide from the uropathogenic *E. coli* K4 bacteria (05:K4:H4) and its defructosylated product [77]. Following chondroitinase digestion of K4 and its derivative, the two disaccharides, ΔHexAFrc-GalNAc for K4 and ΔHexA-GalNAc for defructosylated K4, were separated and determined within 20 min on an uncoated fused-silica capillary using normal polarity and detection at 230 nm (Fig. 9). However, this analytical approach first requires treatment of the bacterial polysaccharide with lyases to produce the relative unsaturated disaccharides. As a consequence, in a further study, a direct HPCE separation using normal polarity of native K4 and K4d polysaccharides has been developed [58]. In fact, the two polyanions were separated and determined within 30 min using normal polarity and detection at 200 nm (Fig. 10). A linear relationship was found for the two polysaccharides over a wide range of concentrations, from approximately 30 to 210 ng.

HPCE was also utilized in our laboratory for the determination of another bacteria polysaccharide produced by the uropathogenic *E. coli* K5 bacteria 010:K5:H4 [59]. This natural polysaccharide having the structure of a desulfo-Hep composed of -4)-βGlcUA-(1,4)-α-GlcNAc-(1- was separated and qualitatively and quantitatively determined within 20 min on an uncoated fused-silica capillary using normal polarity and detection at 200 nm. HPCE was also able to separate several molecular species mainly due to the presence of polysaccharides of distinct and increasing mean chain lengths (Fig. 11). Furthermore, a linear relationship was found for migration time and log molecular mass of different K5 polysaccharide species, and this model was used to calculate the molecular mass of the main K5 species [59].

Lipopolysaccharides (LPSs) from the outer membrane of Gram-negative bacteria are a heterogeneous group of molecules due to the variation of non stoichiometric modifications of the lipid A and core polysaccharides, and to variations in the number of repeat units in the *O*-antigen [78–81]. Several methods have been used to separate the many subclasses of LPS from individual strains, with SDS-PAGE [79,80] and gel-filtration [82] being perhaps the most successful. These methods are, however, hampered by the tendency of LPS to aggregate and by the difficulty in detecting and identifying each distinct subclass. In our laboratory, a rapid, highly sensitive and reproducible HPCE method has been developed for the determination of the LPS and detoxified LPS (D-LPS) from *E. coli* 055:B5 bacteria [83]. LPS and D-LPS were separated and determined within 25 min on an uncoated fused-silica capillary using normal polarity and detection at 200 nm. Furthermore, as also reported for K5 polysaccharide, HPCE was able to separate several molecular species mainly due to the presence of populations with *O*-specific polysaccharides of distinct and increasing mean chain lengths (Fig. 12). This approach could be of great importance for

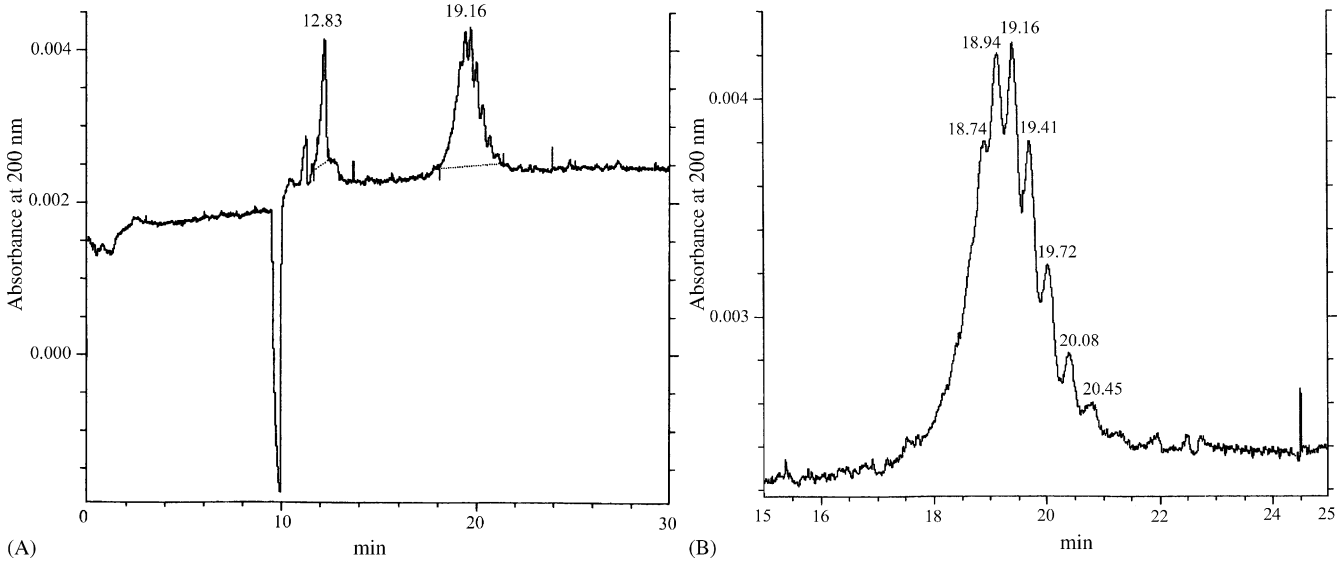

Fig. 12. (A) HPCE electropherograms of *Escherichia coli* 055:B5 LPS at 200 nm. (B) HPCE electropherograms of *Escherichia coli* 055:B5 LPS as in (A) expanded in the region from 15 to 25 min (reprinted with permission from [83]).

the quantitative determination of LPS and D-LPS during purification and preparation processes, also considering the importance of D-LPS in the preparation of human vaccines.

7. Fluorophore-assisted carbohydrate electrophoresis analysis

2-Aminoacridone (2-AMAC) is a fluorescent hydrofobic molecule with an excitation wavelength of 428 nm and an emission wavelength of 525 nm. Quantitative derivatization is generally obtained with a 100-fold excess of this reagent. The 2-AMAC molecule has been used for the derivatization of acidic GAGs-derived di- and oligosaccharides and their subsequent HPCE analysis [25,84].

AMAC characteristics have made it an attractive labeling reagent for analysis of both charged and neutral oligosaccharides with electrophoretic (PAGE and HPCE) and chromatographic techniques. PAGE of AMAC derivatized glycans has been applied to analysis of plant cell wall polysaccharides [85] and HA and GAGs disaccharides [86]. An example of the separation and resolution of the known nonsulfated and monosulfated Δ-disaccharides forming the CS present in normal human plasma is illustrated in Fig. 13. As is evident, FACE was proved in our laboratory to be a powerful analytical technique to detect CS quantity and structure in human plasma [87]. Furthermore, the FACE method was applied for the quantitative analysis of microgram amounts of the *E. coli* K4 bacterium capsule polysaccharide and its defructosylated polymer [88]. In fact, following chondroitinase digestion of K4 and its derivative, the two disaccharides, ΔHexAFru-GalNAc for K4 and ΔHexA-GalNAc for defructosylated K4, were fluorotagged by AMAC and the products separated on a PAGE. The detection limit was found to be approximately 0.5–1 μg for K4 and 0.1–0.2 μg for K4d.

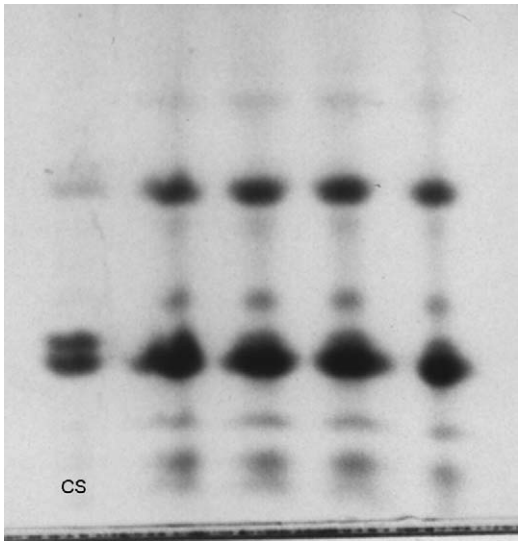

Fig. 13. FACE analysis of human plasma CS. Endogenous CS from a healthy volunteer was extracted from 100 μl of the same plasma sample and repeated four different times. Five microlitre were layered on the gel. CS: standard CS after treatment with chondroitinase ABC and production of unsaturated disaccharides (modified from [87]).

8. Conclusions

GAGs are fundamental macromolecules of many tissues as components of the extracellular matrix but also localized at cellular level, and body fluids, such as plasma and urine. Under physiological conditions, their amount and nature depends on the tissue, organs and species. However, local modifications in their concentration, type and structure have been reported to occur with ageing, but also associated with many diseases. Typical examples are many malignant cases, where a significant modification of GAGs [25] concentration occurs, and the various mucopolysaccharidoses, where increased amounts of DS, HS or KS, are excreted in the urine due to lysosomal enzyme deficiencies. Furthermore, an increase in the amount of a specific polyanion in a tissue or body fluid may be observed in diabetes, renal failure, systemic diseases, meningococcal septicemia, emphysema, etc., causing an increase of GAGs or their products in urine [25]. In these cases, separational techniques must be applied to delineate possible alterations of GAGs, with particular regard to their fine chemical structure, and utilized for the precise diagnosis of the pathophysiological status.

Capsular polysaccharides (K-antigens) and LPS are often present together as antigenic determinants on the surface of Gram-negative bacteria [89]. LPS has been extensively studied by investigators in many disciplines in efforts to elucidate and define relevant pathophysiological parameters of endotoxin shock, a profound life-threatening consequence of bacterial sepsis. Furthermore, the LPS is detoxified by removing the lipid A during the preparation of detoxified-LPS, which may be a useful tool in the study of immunological phenomena related to LPS and has potential as a human vaccine. Actually, the defructosylated product of K4 is useful to gain detailed insight into the mode of action of several enzymes, such as the C-5 epimerase involved in dermatan sulfate biosynthesis [90], and both the GlcA- and the GalNAc-transferases in CS formation [91] while K5 polysaccharide is largely utilized to produce a series of Hep-like products through regioselective chemical sulfatation and enzymatic modification [92–94]. Furthermore, modification of the K5 polysaccharide also provides a useful strategy to study the structure-activity relationship of this class of complex natural polymers [95] and to study the HS/Hep biosynthesis process [96]. As a consequence, polysaccharides are produced by extraction from the bacteria cultures and purified according to different preparation approaches. A rapid, specific and sensitive analytical method to detect these polymers during the extraction and purification procedures is necessary. Furthermore, quantitative responses are important in the light of the modification of the bacteria culture conditions to increase the production of these complex macromolecules. As a consequence, the electrophoretic techniques to the quantitative but also qualitative evaluation of complex polysaccharides, as single macromolecules and in mixtures, and their derived oligosaccharides, are of paramount importance. Furthermore, the presence of negatively charged groups on these (macro)molecules ensures their migration under a variety of conditions with requirement of small sample. These properties, along with the high sensitivity due to a variety of staining procedures, derivatization approaches

and detectors (Table 1) make the electrophoresis separation particularly suitable for the analysis of valuable biological samples.

Electrophoresis on cellulose acetate and nitrocellulose membrane are very useful for the evaluation of GAGs in mixtures and purity of single GAGs, in particular during the extraction and purification processes from tissues or fluids (Table 1). On the other hand, agarose-gel electrophoresis appears to be the most versatile technique due to its capacity to separate several important GAGs, including the two components of Hep, SMHep and FMHep, and to further evaluate their properties. In fact, GAGs may be separated by means of agarose-gel electrophoresis and single species further recovered for the determination of the molecular mass and disaccharide pattern [51] but also blotted and immobilized on membranes [52] and immunodetected by specific antibodies [53]. Furthermore, due to the very low limits of detection, approximately 0.001–0.01 μg GAGs performed by several staining procedures (Table 1), and its ability to detect nonsulfated macromolecules, such as HA or bacterial polysaccharides, agarose-gel electrophoresis has been applied for the characterization of GAGs extracted from various organs, tissues and cells, and biological fluids, to identify modifications due to pathological conditions, and to measure quantitatively purified GAGs and their mixtures used as drugs.

HPCE is able to separate nonsulfated GAGs, i.e. HA and bacterial polysaccharides, as such as disaccharides and oligosaccharides produced by the action of enzymes [23–26] (Table 1). Furthermore, HPCE is a powerful analytical technique due to the extremely small amount of sample used and to the high-resolution separation, characterization, and reproducible quantitation of analytes also for the possibility to label carbohydrates with chromophores or fluorophores [25,26].

FACE is a simple procedure able to separate GAGs-derived disaccharides and oligosaccharides (Table 1) by means of a high-yield chemical step capable to provide anionic and intensely fluorescent derivatives. As a consequence, FACE separation has been utilized for the fine characterization of GAGs extracts of cells, tissues and biological fluids in normal and pathological conditions [86,87,97], as such as it is considered important for routine use in monitoring disease and biopharmaceuticals.

In conclusion, a variety of electrophoretic methods have been developed for the analysis of acidic (poly)saccharides. These methods allow on direct detection or pre-derivatization for both qualitative and quantitative analysis with a high level of sensitivity. However, new and recent methods, essentially based on gel or capillary electrophoresis coordinated in particular with mass spectrometric approach, will permit the development of new strategies for the rapid fingerprinting and structure identification of (oligo)saccharides.

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