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# Analysis of fluorophore-labelled hyaluronan and chondroitin sulfate disaccharides in biological samples

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**Abstract**

In this report, we describe a polyacrylamide gel electrophoresis for the analysis of fluorophore-labelled hyaluronan (HA) and chondroitin sulfate (CS) Δ-disaccharides. The method utilizes derivatization of reducing ends of hyaluronan and the variously sulfated chondroitin sulfate Δ-disaccharides with 2-aminoacridone (AMAC), followed by electrophoresis in Tris–HCl buffer (pH 8.8), and polyacrylamide gel (*T* 25%/*C* 3.75%). The method was applied to the analysis of GAGs secreted into the culture medium of human aortic smooth muscle cells and the obtained results were compared with those analysed by fluorophore assisted carbohydrate electrophoresis (FACE). The described method is a useful and sensitive tool for the rapid monitoring of GAGs in high number of biologic samples.


*Keywords:* Disaccharides; Chondroitin sulfate; Hyaluronan; Aortic smooth muscle cells; Polyacrylamide gel electrophoresis

## 1. Introduction

Glycosaminoglycans (GAGs) are linear heteropolysaccharides composed of repeating disaccharide units. Hyaluronan (HA), chondroitin sulfate (CS) and dermatan sulfate (DS) are among the major GAGs present in the extracellular matrix. CS, usually referred to as galactosaminoglycan (GalAG), consists of repeating disaccharides units of [→ 4GlcAβ1 → 3GalNAcβ1 →]. It is highly charged chain due to the presence of esterified groups at any available hydroxyl group of the disaccharide [1]. CS sulfated at C-4 and C-6 position of GalNAc is predominant GAGs in the ECM of the arterial wall and the modification of Δdi-mono4S/Δdi-mono6S ratio plays a critical role in diseases [2–5]. HA, on the other hand, is composed of the repeating disaccharide unit [→ 4GlcAβ1 → 3GlcNAcβ1 →]. Its structure differs from that of CS in that the GalNAc is replaced with GlcNAc. It forms aggregates with proteoglycans (PGs) of high molecular size.

Gel electrophoresis is a method of choice in routine analysis of GAGs. Electrophoresis of fluorophore-labelled saccharides has been previously described

*Abbreviations:* AMAC, 2-aminoacridone; AoSMCs, human aortic smooth muscle cells; CS, chondroitin sulfate; Δdi-nonSHA, non-sulfated disaccharides derived from HA; Δdi-nonSCS, non-sulfated disaccharides derived from CS; Δdi-mono4S, monosulfated disaccharides at C-4 of galactosamine; Δdi-mono6S, monosulfated disaccharides at C-6 of galactosamine; Δdi-mono2S, monosulfated disaccharides at C-2 of uronic acid; GAGs, glycosaminoglycans; HA, hyaluronan

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for analysis of N-linked glycans of mammalian [6], as well as fluorophore assisted carbohydrate electrophoresis (FACE) for analysis of total HA and CS [7,8] and polysaccharide analysis using carbohydrate gel electrophoresis (PACE) for analysis of plant polysaccharides [9]. Gel electrophoresis is relatively economical, fast and easy method. Many samples can be analysed in parallel, giving it the potential for a high throughput. Quantitation of the total amount of GAGs is determined when standards are analysed in the same gel.

In this report, we describe a technique that involves derivatization of the unsaturated disaccharides with the fluorophore 2-aminoacridone (AMAC), followed by electrophoresis in polyacrylamide gels. The method was applied to the analysis of HA and CS isolated from the culture medium of human aortic smooth muscle cells and compared with the FACE analysis.

## 2. Materials and methods

### 2.1. Chemicals

HA Δ-disaccharides ($Na^+$ salt) and variously sulfated CS Δ-disaccharides ($Na^+$ salt) were purchased from SIGMA (St. Louis, MO, USA). Protease K (EC 3.4.21.64) was from FINNZYMES and hyaluronidase SD (EC 3.2.1.35) and chondroitinase ABC (EC 4.2.2.4) were from Seikagaku Kogyo (Tokyo, Japan). AMAC was obtained from Molecular Probes (Oregon, USA) and $NaBH_3CN$ from Sigma–Aldrich (Steinheim, Germany). Tris (hydroxymethyl)-aminomethane was from FLUKA Chemie (Bucks, Switzerland) and acetonitrile from Merch (Darmstadt, Germany). Acrylamide and *N*,*N*′-methylenebisacrylamide were obtained from Bio-Rad (Richmond, CA), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) from BDH Chemicals (Poole, England) and ammonium persulfate from LKB (Bromma, Sweden). FACE® Monosaccharide composition kit from GLYKO (Novato, CA). All other chemicals used were of analytical reagent grade.

### 2.2. Derivatization procedure

Derivatization of standard HA and CS Δ-disaccharides was performed as described by Calabro et al. [8]. In particular, 10 nmol of each Δ-disaccharide standard in water was completely evaporated in a microcentrifuge tube at 11,000 × *g* at room temperature. A 40 μl volume of AMAC solution (12.5 mM) in glacial acetic acid–DMSO (3:17, v/v) was added and samples were incubated for 10–15 min at room temperature. A 40 μl of a freshly prepared solution of $NaBH_3CN$ (1.25 M) in water was added to each sample, followed by an overnight incubation at 37 °C.

### 2.3. Electrophoresis

A MINIPROTEAN II cell vertical slab gel electrophoresis apparatus (Bio-Rad) was used with 7.2 cm plates, 0.75 mm spacer, and well of 0.5 cm. The stock buffer solutions were Tris–borate (pH 8.8; 1.5 M) and Tris–HCl (pH 8.8; 1.5 M). Acrylamide solution *T* 50%/*C* 7.5% for the resolving gel and *T* 50%/*C* 15% for the stacking gel were used as stock solutions [% *T* refers to the total concentration (w/v) of acrylamide monomer (i.e. acrylamide plus methylenebisacrylamide); % *C* refers to the concentration (w/v) of cross-linker relative to the total monomer].

A 10 ml volume of *T* 25%/*C* 3.75% resolving gel solution, in Tris–HCl (pH 8.8; 0.375 M) was prepared and degassed. A 5 μl volume of TEMED and 50 μl of a freshly prepared 10% (w/v) ammonium persulfate were added. The solution was mixed rapidly and then placed between the glass plates, avoiding the air bubbles. The non-polymerized gel was overlaid with butanol. Polymerization occurred within ∼20 min. The gel can be used immediately or stored at 4 °C for 1–2 weeks.

The resolving gel surface was rinsed with stacking gel buffer (Tris–HCl diluted from the stock solution). A 5 ml of *T* 5%/*C* 1.5% acrylamide stacking gel in Tris–HCl (pH 8.8; 0.36 M), was prepared, followed by an addition of 5 μl of TEMED and 50 μl of 10% ammonium persulfate. Immediately the solution was poured on the top of the resolving gel the well-forming comb was inserted. The height of the stacking gel was 5 mm and the polymerization occurred ∼45 min.

Immediately before the electrophoresis, the wells were rinsed with electrophoresis buffer. A pre-run of the gel was performed in 400 V at 4 °C for 10 min. A 5 μl volume of each sample, supplemented with glycerol in a final concentration of 20% (v/v), was

loaded in each well. A marker sample containing bromophenol blue was also run. Electrophoresis was done at 400 V and 4 °C and terminated when the marker dye was 1.2 mm from the bottom of the gel (~45 min).

### 2.4. Gel imaging and product quantitation

Gels were scanned in a UV-light box using a CCD camera (Gel Doc 2000 System) from Bio-Rad Laboratories (Hercules, CA). Identification and quantification of sample bands were done by comparing their migration and the pixel density with standard Δ-disaccharides, running in the same gel.

### 2.5. FACE analysis (fluorophore assisted carbohydrate electropheresis)

A 5 μl volume of each sample, supplemented with glycerol in a final concentration of 20% (v/v), was analysed using a FACE® Monosaccharide composition kit. The analysis was carried out at 500 V, at 4 °C for 50 min. The gel was visualized by exposure to UV-light, as described before.

### 2.6. Cell culture

Human aortic smooth muscle cells (AoSMCs) and their growth medium Bulletkit-2 (SmGM-2) were obtained by the Clonetics™ (BioWhittaker) as well as all the other reagents. Cells ($10^5$) were seeded in a T-25 flask and the growth medium was changed daily. The cells subcultured at early confluence using trypsin/EDTA. The trypsin was inactivated with trypsin neutralize solution (TNS) and the number of cells in suspension was determined using Burker chamber. For the experiments, a number of $50 \times 10^4$ cells was seeded in a well plate and the culture was maintained at 37 °C (5% $CO_2$ in air with 90% humidity) in SmGM-2 medium. The medium was changed daily and harvested the fifth day.

### 2.7. Isolation and degradation of GAGs

Medium was frozen in −80 °C and lyophilised in a microcentrifuge tube at room temperature. A 300 μl volume of deionised water and 96% ethanol (or absolute ethanol) in a ratio of 1:4, was added in the sample and the mixture was kept at −20 °C overnight. Following centrifugation at 11,000 × *g* at 4 °C for 15 min, the pellet was left to dry. The obtained pellet was dissolved in 300 μl of ammonium acetate buffer (pH 7.0, 100 mM) containing 20 U/ml of protease K and digested in 60 °C for 2 h. The reaction was terminated by heating at 100 °C for 5 min. Thereafter, 4 vol. of 96% ethanol per sample volume were added, and the mixture was stored at 20 °C overnight.

Ethanol precipitated GAGs were centrifuged at 11,000 × *g* at 4 °C for 15 min. The obtained pellets were dried and dissolved in 100 μl of ammonium acetate (pH 7.0; 100 mM) containing 100 mU/ml of hyaluronidase SD and digested in 37 °C for 1 h. A 100 mU/ml of chondroitinase ABC was added, and the mixture was incubated at 37 °C for 3 h. The samples were then frozen at −80 °C and then lyophilised. The Δ-disaccharide digested products were then derivatized as described above.

## 3. Results and discussion

It has already been reported that derivatization of HA and CS Δ-disaccharides with AMAC improves dramatically the detection sensitivity of various separation techniques, such as HPLC, FACE and CZE [10–12]. Derivatization with AMAC has been recently used to study polysaccharides found in plant walls [9]. This method allows the labeling of the reducing ends of unsaturated disaccharides obtained after enzymic degradation of GAG chains. The amino group of AMAC reacts with carbonyl group of the reducing end of disaccharide moiety to form a Schiff base, further reduced with sodium cyanoborohydride to form a stable secondary amine [13]. AMAC derivatives are stable for months when they are stored at −80 °C [14].

Optimization of gel electrophoresis was performed for Δ-disaccharides of HA and CS sulfated in various hydroxyl groups. The gels used had always a stacking and a resolving gel. The stacking is important as it facilitates the uniform entry of samples into the resolving gel and furthermore retards the excess AMAC. Following pilot experiments it was found that the optimal height of the stacking and resolving gel were 5 mm and 5.5 cm, respectively.

In order to optimize the electrophoretic conditions, various running conditions were tested. These involved the percentage of acrylamide in the gels, the concentration of *N*,*N*′-methylenebisacrylamide in the gels and the type of electrophoresis buffers. All values of acrylamide concentration in the resolving gel between 25 and 30% separated well the disaccharides tested. It was worth noticing that cross-linker played a crucial role for effective separation. In particular, only the *C* 3.75% ensured a well separation of the disaccharides. They were also examined different concentration of cross-linker in the stacking gel. To sum up, a *T* 25%/*C* 3.75% of resolving gel and *T* 5%/*C* 1.5% of stacking gel ensured the best separation of the examined Δ-disaccharides.

Different types of buffer were used for stacking and resolving gels and for the electrophoresis. Turnbull and Gallagher [15] have demonstrated that Tris–HCl buffer in the gels can separate highly sulfated heparan sulfate oligosaccharides. Based on this demonstration, the same buffer was used for the preparation of both of the gels. The separation between sulfated disaccharides was optimum, but the separation of non-sulfated disaccharides was poor. To improve the last separation, a Tris–borate buffer was used as a running buffer. Lamari and Karamanos [16] have already demonstrated that using Tris–borate buffer in capillary electrophoresis analysis, the non-sulfated disaccharides were completely separated (Fig. 1, lane 1).

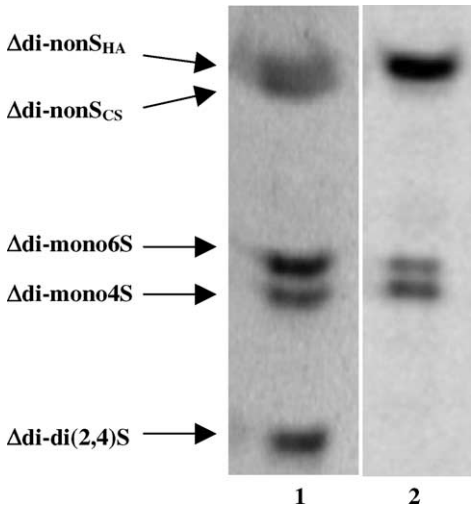


Fig. 1. Analysis of AMAC-derivatives of HA and variously sulfated CS Δ-disaccharides. The stacking gel consisted of polyacrylamide *T* 5%/*C* 1.5% in a 0.36 M Tris–HCl buffer, pH 8.8, and the running buffer was 0.15 M Tris–borate, pH 8.8. The resolving gel consisted of *T* 25%/*C* 3.75% with 0.375 M Tris–HCl, pH 8.8 as resolving buffer. Lane 1: profile showing the separation of standard HA and CS-derived AMAC derivatized disaccharides. Lane 2: profile obtained for GAGs isolated from the culture medium of AoSMCs.

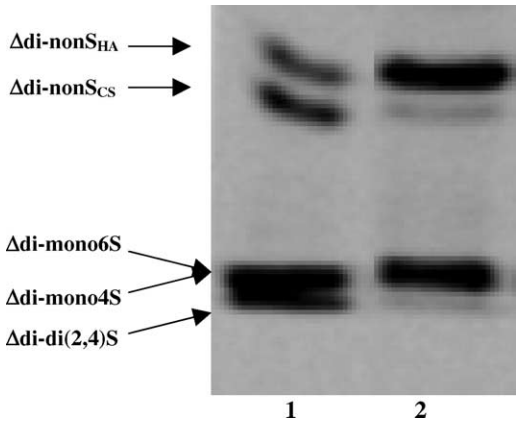


Fig. 2. Analysis of AMAC-derivatized Δ-disaccharides using polyacrylamide gels, developed by GLYKO. The electrophoresis was carried out at 500 V at 4 °C for 50 min. Lane 1: profile showing the separation of standard HA and CS-derived AMAC derivatized disaccharides. Lane 2: profile obtained for GAGs isolated from the culture medium of AoSMCs.

The method was applied to analyze HA and CS Δ-disaccharides secreted to the medium of human AoSMCs, as described in Material and Methods. Results from analysis showed that HA was particularly abundant in the medium, accounting for 70% of the total amount of GAG (Fig. 1, lane 2). In particular, the amount of Δdi-nonSHA, Δdi-mono4S and Δdi-mono6S in 5 μl of sample was 384, 120 and 57 pmol, respectively. CS present in the culture medium is mainly sulfated at C-4, which is in agreement with previous data [5].

A mixture of standard HA and CS Δ-disaccharides and the obtained Δ-disaccharides from the medium of AoSMCs were analysed also by FACE (Fig. 2). The amount of Δdi-nonSHA in 5 μl of sample was found to be 381 pmol, which is in agreement with that of the studied gel. The sulfated CS Δ-disaccharides were not analysed quantitatively as the separation was not efficient (Fig. 2, lane 2).

## 4. Conclusions

In this report we described a powerful and flexible PAGE method to analyze HA- and CS-derived disaccharides after their derivatization with the fluorophore AMAC. The procedure was simple and rapid since twenty samples can be analyzed in one run (two minigels in one apparatus), requiring only 2 h for gel preparation and ca. 1 h for electrophoresis. Moreover, this technique is of low cost since it does not require expensive equipment, as other approaches for carbohydrate analysis. It is a fast method for estimating the total amount of HA and various sulfated CS disaccharides in a small quantity of material. The applicability was examined by analyzing successfully the GAG-derived Δ-disaccharides from the culture medium of a human aortic smooth muscle cell cultures. This developed technique shows an efficient separation between sulfated Δ-disaccharide. FACE analysis developed by GLYKO, however, cannot separate them well and the quantitative analysis is impossible. The data obtained suggest that the described method may be a useful tool for a fast screening, particularly when a high number of samples should be analyzed.

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## References

[1] N.K. Karamanos, A. Syrokou, P. Vanky, M. Nurminen, A. Hjerpe, Anal. Biochem. 221 (1994) 189–199.
[2] A.D. Theocharis, I. Tsolakis, T. Tsegenidis, N.K. Karamanos, Atherosclerosis 145 (1999) 359–368.
[3] A.D. Theocharis, D.A. Theocharis, G. De Luca, A. Hjerpe, N.K. Karamanos, Biochimie 84 (2002) 667–674.
[4] R. Albertini, P. Ramos, A. Giessauf, A. Passi, G. De Luca, H. Esterbauer, FEBS Lett. 403 (1997) 154–158.
[5] R. Albertini, G. De Luca, A. Passi, R. Moratti, P.M. Abuja, Arch. Biochem. Biophys. 365 (1999) 143–149.
[6] P. Jackson, Mol. Biotechnol. 5 (1996) 101–123.
[7] A. Calabro, V.C. Hascall, R.J. Midura, Glycobiology 10 (2000) 283–293.
[8] A. Calabro, R. Midura, A. Wang, L. West, A. Plaas, V.C. Hascall, Osteoarthritis Cartilage 9 (Suppl. A) (2001) S16–S22.
[9] F. Goubet, P. Jackson, M.J. Deery, P. Dupree, Anal. Biochem. 300 (2002) 53–68.
[10] G. Okafo, J. Langridge, S. North, A. Organ, A. West, M. Morris, P. Camilleri, Anal. Chem. 69 (1997) 4985–4993.
[11] A. Calabro, M. Benavides, M. Tammi, V.C. Hascall, R.J. Midura, Glycobiology 10 (2000) 273–281.
[12] F. Lamari, A. Theocharis, A. Hjerpe, N.K. Karamanos, J. Chromatogr. B 730 (1999) 129–133.
[13] T.N. Mitropoulou, F. Lamari, A. Syrokou, A. Hjerpe, N.K. Karamanos, Electrophoresis 22 (2001) 2458–2463.
[14] M. Militsopoulou, F.N. Lamari, A. Hjerpe, N.K. Karamanos, Electrophoresis 23 (2002) 1104–1109.
[15] J.E. Turnbull, J.T. Gallagher, Biochem. J. 251 (1988) 597–608.
[16] F. Lamari, N.K. Karamanos, J. Liq. Chromatogr. Rel. Technol. 22 (1999) 1295–1317.