Improved and simple micro assay for sulfated glycosaminoglycans quantification in biological extracts and its use in skin and muscle tissue studies

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This article describes a simple and selective procedure used for direct measurement of sulfated glycosaminoglycans (GAGs) in biological samples and its application to the determination of GAGs during tissue regeneration and myogenic differentiation. We describe a modified procedure of previous GAG assays that has improved specificity, reproducibility, and sensitivity. The assay is based on the ability of sulfated GAGs to bind the cationic dye 1,9-dimethylmethylene blue. We describe conditions that allow isolation of the GAG–dye complex. This complex was dissociated; the optical density measurement of the dissociated dye permitted quantification of GAGs in biological samples. Applied to the study of myogenic cell differentiation in vitro, muscle repair, and skin ulceration, this method revealed significant modifications in the patterns of expression of different sulfated GAGs in these tissues. In particular, application of the method after nitrous acid treatment revealed that heparan sulfate and chondroitin sulfate ratio changed during muscle regeneration process.

Key words: cell culture/1,9-dimethylmethylene blue/glycosaminoglycans/skeletal muscle/skin

Introduction

Glycosaminoglycans (GAGs) are one of the major macromolecular components constituting the cellular environment. Except for hyaluronic acid, all GAGs possess N- and/or O-sulfate groups distributed on their disaccharide building blocks. Most sulfated GAG chains are covalently linked to core proteins to form proteoglycans, which are strategically located at the cell surface and in the extracellular matrix (Bernfield et al., 1999; Kato et al., 1994). In addition to their structural functions, proteoglycans and GAGs play different roles in diverse processes, such as enzyme regulation and cellular adhesion, growth, migration, or differentiation (Lyon and Gallagher, 1998; Selleck, 2000; Turnbull et al., 2001). These processes are mediated by their binding capacities and focal sequestering of a number of biologically active proteins (David and Bernfield, 1998). Among them, growth factors may be causal for many biological events, such as cell activation in response to injury. The knowledge of GAG content and composition after tissue injury is therefore of particular importance in the understanding of processes involved in reconstitution of the mature phenotype.

Several spectrophotometric assays are available in literature to measure levels of sulfated GAGs in biological samples. Many of these assays are based on changes in the absorption spectrum of the dye 1,9-dimethylmethylene blue (DMMB) when bound to GAGs (Whitley et al., 1987; Chandrasekhar et al., 1987; Farndale et al., 1982). Although recent studies have demonstrated the efficiency of DMMB over other assays (toluidine blue, Alcian blue, etc.), we have found that reported DMMB methods suffer from multiple drawbacks. The reagent used is unstable, interferences with DNA or other negatively charged molecules seriously modify the response, and, in addition, the GAG–dye complex is not stable in solution, and GAG measurements are not accurate because of this instability. Indeed, variations of the time between sample measurements may modify results (Farndale et al., 1982; Panin et al., 1986; Stone et al., 1994). Furthermore, accuracy, precision, and linearity are poor and even unacceptable at levels of less than 5 µg/ml of GAGs.

In this article we report a modified procedure for the use of DMMB that overcomes these problems. Our assay is based on the generation of an insoluble precipitate of the GAG–DMMB complex. We present conditions allowing the formation of a solid GAG–dye complex that was stable for at least 1 h, which allows its isolation from other sample constituents, including other biological molecules and excess of DMMB. Linearity, precision, and accuracy of the assay were validated at concentrations of GAGs ranging from 0.025 to 10 µg/ml in biological samples. We have applied this method to the study of the total sulfated GAG content in cultured cells and tissue extracts. The method was also used to discriminate the contents of heparan sulfate (HS) from chondroitin sulfate (CS) in these biological materials after nitrous acid treatment. Our results showed relevant modifications in the amounts of total sulfated GAG and in their composition during skeletal muscle repair, in differentiating myogenic cell cultures, and in ulcerated skin.
Results and discussion

Isolation of the GAG–DMMB complex

Several spectrophotometric assays for sulfated GAG quantification in biological samples based on the use of DMMB have been reported (Chandrasekhar et al., 1987; Farndale et al., 1982; Whitley et al., 1989). All of these methods rely on the absorption at about 525 nm of the GAGs–DMMB complex formed after addition of the dye to biological samples. In all reported methods, this complex is formed in a reaction mixture containing a large excess of the dye, which absorbs in wavelengths that might interfere with the detection of the GAG–dye complex (Carroll, 1987; Chandrasekhar et al., 1987; Farndale et al., 1982, 1986; Klompmakers and Hendriks, 1986; Panin et al., 1986; Stone et al., 1994; Templeton, 1988; Whitley et al., 1989).

Among other drawbacks of DMMB classical methods, the presence of a precipitate of the GAG–dye complex has been reported to render quantification difficult (Chandrasekhar et al., 1987; Stone et al., 1994).

To overcome these drawbacks, we decided to isolate the GAG–DMMB complex from soluble materials, including DMMB excess. Therefore we developed conditions that increased complexation of dye to sulfated GAGs by using a DMMB solution prepared in a formate buffer pH 3.0 containing 5% ethanol and 0.2 M guanidine hydrochloride (GuHCl). The use of ethanol at low pH allowed exhaustive complex precipitation within 30 min when vigorous shaking was applied during this time. The presence of GuHCl has already been reported by others to avoid interactions with negatively charged macromolecules other than GAG during the complexation process. The GAG–DMMB complex was obtained as a stable pellet after centrifugation of the treated sample, and its isolation was particularly easy by simple decantation of soluble materials.

The next step consisted of the dissociation of the complex to render DMMB soluble. The UV absorption of the decomplexed DMMB is then proportional to the GAG amount complexed from the original sample. This step was achieved by using a decomplexation 4 M GuHCl solution at pH 6.8 containing 10% propan-1-ol. Dissociation was favored at neutral pH because the cationic state of the dye, which induced complexation to polyanionic GAGs, was highly reduced at this pH. The high GuHCl concentration allowed GAG solubility favoring its dissociation from the complex. We included 10% propan-1-ol in the decomplexation solution to enhance DMMB signal at 656 nm. This wavelength became the most sensitive to changes in dye concentration and was thus selected for quantification after decomplexation (Figure 1a and 1b). As an example, when different amounts of CS were treated by the DMMB complexation/decomplexation protocol, two peaks of absorption, at about 610 nm and 656 nm, were detected in samples (Figure 1d). The absorbance of the highest peak (656 nm) was proportional to the amount of GAG present in the original sample (see method validation). It must be kept in mind that in some classical methods, GAGs are evaluated by ratio determinations of the absorbance at about 525 nm (complex signal) versus 595 or 652 nm (noncomplexed DMMB signal). When vigorous agitation and complex isolation steps were omitted from our complexation/decomplexation protocol, the UV spectrum of the complexed sample showed an additional peak (shoulder) at 525 nm resulting from the absorption of the GAG/dye complex (Figure 1c).

Stability of the DMMB solution

One drawback mentioned by several authors using DMMB dye assay was the instability of the solution during its...
storage. When we prepared a solution at 16 mg/L, as in classical methods, precipitation of material indeed occurred few hours after preparation. We interpreted this as a consequence of a low solubility of DMMB in the formate buffer; small DMMB aggregates behaved as seeds for promoting precipitation. Addition of ethanol and GuHCl to this reagent solution did not improve this solubility. However, when using a chemist strategy for solubilization, which consists of the preparation of a 32 mg/L solution followed by twofold dilution in the ethanol/GuHCl formate buffer, the DMMB solution obtained was stable for more than 4 months when stored at room temperature in the darkness. It can be supposed that DMMB, partially aggregated at the double final concentration, disaggregates into single molecules in the final diluted solution.

**Condition for minimizing DNA–DMMB interaction in DNA-containing samples**

Biological samples are supposed to contain DNA. Samples were presently treated by proteinase K before the complexation step as described in *Materials and methods*. This treatment dissociated GAGs from protein cores and at the same time liberated DNA from nuclear proteins. It has been reported that, like sulfated GAGs, DNA readily forms complexes with the dye, absorbing at nearly the same wavelength as the GAG–dye complex. Some authors suggested the use of GuHCl (up to 0.24 M) in the complexation solution to decrease these DNA/dye interactions (Chandrasekhar et al., 1987). Accordingly, our results show that low GuHCl concentration (0.08 M) did not avoid interaction of DNA with DMMB and thus disturbed GAG determination (Figure 2). Another way to overcome DNA–dye interaction was to eliminate or highly reduce DNA by filtration of samples after proteinase K treatment. Filtration of the preheated proteinase K–treated sample proved to eliminate most of the interfering DNA, which was no longer detected by complexation with DMMB even in the absence of GuHCl. Our results show that GuHCl concentration may be increased to 0.20 M without affecting GAG–DMMB complexation (Figure 2) as recommended by Chandrasekhar et al. (1987). The proteinase K treatment and the filtration procedure did not affect CS concentration of standard solutions.

**Validation of sulfated GAG quantification**

The method for sulfated GAG determination was validated to ascertain that it was reproducible, sensitive, accurate, and linear, at a specified range of concentrations that correspond to GAG contents generally found in biological samples, providing an assurance of reliability. Validation of the method was performed with muscle extracts to which known amounts of CS (1–5 µg/ml) were added (spiked extracts) and with standard solutions of CS or HS prepared and processed as described in *Materials and methods*. GAG amount was measured repeatedly and found to be stable in proteinase K–treated samples stored at −20°C for several months. Precision, defined as the degree of repeatability of the analytical method and expressed as the coefficient of variation (CV) corresponding to the percent relative standard deviation for a statistically significant number of samples (*N* = 5), was found to be inferior to 10% (Table I). The limit of GAG quantification in biological samples, defined as the lowest amount measurable with a CV inferior to 15%, was under 0.025 µg/ml. This limit of quantification is superior to other DMMB methods. Recently, a method for detection of submicrogram quantities of GAG on agarose gel electrophoresis has been reported using sequential staining with toluidine blue and Stains-all. Whereas this method is more laborious than ours, it is complementary because it can be applied to nonsulfated polysaccharide quantification (Volpi and Maccari, 2002).

Accuracy, defined as the measure of the method exactness, was expressed as the relative percentage of recovery from the nominal sample concentration obtained by analyzing biological samples (*N* = 5) before and after spiking with CS standard solutions. It was between 89% and 102% in spiked muscle samples (Table I). In spiked skin samples, accuracy ranged from 83% to 89%.

Linearity was ascertained by calibration curves. Because extraction recoveries from GAG-spiked tissue extracts and

![Fig. 2. Effects of GuHCl and sample filtration on the interaction between DNA and DMMB. DNA in biological extract contained 2 µg DNA as determined by the DAPI method. Pure DNA solution contained also 2 µg of the nucleic acid.](image)

<p>| Table I. Accuracy and precision of DMMB method for determination of GAG in biological samples (<em>N</em> = 5) |</p>
<table>
<thead>
<tr>
<th>GAG quantity</th>
<th>Nominal (µg)</th>
<th>Found (µg)</th>
<th>Accuracy (% recovery)</th>
<th>Precision (CV %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal*</td>
<td>5.41</td>
<td>5.41</td>
<td>100.0</td>
<td>1.47</td>
</tr>
<tr>
<td>1.35*</td>
<td>1.28</td>
<td>94.8</td>
<td>4.77</td>
<td></td>
</tr>
<tr>
<td>2.70*</td>
<td>2.75</td>
<td>101.8</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>6.41*</td>
<td>6.12</td>
<td>95.5</td>
<td>7.70</td>
<td></td>
</tr>
<tr>
<td>7.41*</td>
<td>6.67</td>
<td>90.0</td>
<td>9.41</td>
<td></td>
</tr>
<tr>
<td>9.41*</td>
<td>8.42</td>
<td>89.5</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>10.41*</td>
<td>9.37</td>
<td>90.0</td>
<td>1.99</td>
<td></td>
</tr>
</tbody>
</table>

*Mean of five measures.

*Precision: CV of the relative standard deviation (*N* = 5).

*Basal quantities of GAGs obtained in a biological extract after proteinase K digestion and filtration. The same sample was used for validation studies.

*Basal nonspiked sample diluted at 25%.

*Basal nonspiked sample diluted at 50%.

*CS (1–5 µg) spiked basal sample.
extraction recoveries from standard solutions were comparable, calibration curves were included into each assay performed with appropriate diluted standard solutions. Figure 3 shows the linear regression curves obtained by plotting the absorbance values of DMMB in solution after decomplexation from CS versus its nominal ng or μg/ml concentration. For submicrogram quantities of GAGs (0.025–1.1 μg/ml), the calibration curve (a) was used (Figure 3a). For samples containing microgram quantities of GAG (0.5–5 μg/ml), the calibration curve (b) was applied (Figure 3b). Linearity at both low and high quantities of GAGs was excellent (R² = 0.9919 and R² = 0.9931, respectively) and was demonstrated up to 10 μg/ml (data not shown).

The reliability of our method had prompted us to use this complexation/decomplexation protocol for applications other than GAG quantification in biological samples. For example, we used it as a method for rapid extraction of GAGs, which were further used for various applications. The present technique permitted the measurement of immobilized natural or synthetic sulfated polysaccharides and the quantification of synthetic GAG analogs (unpublished data).

**Analysis of sulfated GAGs in different biological samples**

The DMMB complexation/decomplexation protocol allowed quantification of sulfated GAGs in cell and in tissue extracts. Tables II and III show amounts of GAGs in myogenic cell cultures and in intact or injured tissues. The method also allowed GAG quantification after nitrous acid treatment, which discriminated between HS and CS in the biological samples. Standard deviations were found to be optimal (at most 13%) when three determinations were performed on the same tissue extract. Results have shown that total sulfated GAG amount increased when myoblasts differentiated into myotubes. During *in vivo* muscle regeneration, we found that total sulfated GAG content highly decreased during the myolysis phase (day 1). It increased from day 3 after the crush, when newly formed myotubes appeared. This increase might in part be due to GAG synthesis by myogenic cells, which regenerate myofibers. Indeed, GAG produced by myoblasts increased on myogenic differentiation *in vitro* (Table II).

Interestingly, at day 9 after crush, total sulfated GAG content was about three times higher than in intact animals. This increase correlated in time with the beginning of innervation of newly formed fibers in regenerating muscle (Bassaglia and Gautron, 1995). In addition, changes in qualitative GAG composition (HS versus CS) occurred during muscle regeneration. These observations might be relevant because it has been demonstrated that expression of some proteoglycans are necessary for the modulation of myogenesis (Jenniskens et al., 2000, 2002; Larrain et al., 1997a,b; Olwin and Rapraeger, 1992). GAGs probably act as low-affinity receptors for several growth factors (for a review, see Husmann et al., 1996), including basic fibroblast growth factor (Olwin and Rapraeger, 1992; Papy-Garcia et al., 2002), hepatocyte growth factor (Lyon and Gallagher, 1998), or transforming growth factor β-1, which are known to regulate myogenesis (Lopez-Casillas et al., 2003; Riquelme et al., 2001). Proteoglycans have also been implicated in the induction of postsynaptic differentiation (Mars et al., 2000; Peng et al., 1995).

In the skin, although the proportion of sulfated GAGs (HS and CS) was not changed, a decrease in the amount of total sulfated GAG was observed when the skin reached a maximum of ulceration. To our knowledge, there is no

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**Table II. Sulfated GAGs in biological extracts**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Sulfated GAG</th>
<th>HS</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/μg ADN</td>
<td>SD (%)</td>
<td>μg/μg ADN</td>
</tr>
<tr>
<td>Myoblasts</td>
<td>3.72</td>
<td>0.181 (4.87)</td>
<td>1.07</td>
</tr>
<tr>
<td>Myotubes</td>
<td>6.79</td>
<td>0.107 (1.57)</td>
<td>5.32</td>
</tr>
</tbody>
</table>
available data on GAG content in this model. However, GAG content has been reported to be decreased in the skin of diabetic rats (Cechowska-Pasko et al., 1999).

Altogether, we believe that the present method based on the isolation of sulfated GAG–DMMB complex will contribute to more efficient studies concerning the physiological evolution of the different GAGs in biological processes, particularly during tissue repair. We are currently working in this area.

**Materials and methods**

**Materials**

Sodium acetate, sodium formate, formic acid, and proteinase K were from Merck (Darmstadt, Germany), and propan-1-ol was from Prolabo-VWR (Strasbourg, France). Filters Ultrafree-MC Amicon was from Millipore (Bedford, MA). All other chemicals, including DMMB, were from Sigma (St. Louis, MO). Cell culture products were from Gibco BRL (Cergy-Pontoise, France). Pentobarbital was from Sanofi-Synthelabo (Libourne, France). Myoblast cell line C2.7, isolated from mouse skeletal muscle, was initially established by Pinset et al. (1988). Wistar rat and Swiss mice were provided by Janvier (Le Genest St. Isle, France). Doxorubicine chlorhydrate was from Teva Pharma S.A. (Courbevoie, France). Depilatory Veet cream came from Reckitt Benckise (Massy, France). Spectroscopic data were collected using a scanning spectrophotometer PU 8740 UV/Vis (Phillips, Paris, France). For vigorous agitation of samples a vortex-Genie with a plate (Scientific Industries, USA) was used.

**Preparation of DMMB solution**

Preparation of the DMMB solution was derived from that reported by Farndale et al. (1982) with some modifications. In brief, 16 mg DMMB were dissolved in 25 ml ethanol and filtered through filter paper. One hundred milliliters of 1 M GuHCl, 1 g sodium formate, and 1 ml 98% formic acid were then added to the DMMB ethanolic solution, and the final volume was completed to 500 ml with distilled water. This solution was unstable and needed to be rapidly diluted (1:1) with the formate solution prepared as described but without DMMB. This DMMB solution was stable for at least up to 4 months when stored at room temperature in the darkness.

**DMMB decomplexation solution**

A 50 mM sodium acetate solution buffer (pH 6.8) containing 10% propan-1-ol was prepared and used to solubilize powdered GuHCl to a final concentration of 4 M. This solution was stable for at least 4 months at room temperature.

**Biological materials**

**Cell culture.** Mouse myoblast cell line C2.7 was maintained as subconfluent monolayers in Dulbecco modified Eagle medium (DMEM) containing 1% glucose and 4 mM L-glutamine supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, and 10 μg/ml streptomycin. Cell cultures were incubated at 37°C in 10% CO2. Samples of proliferating cells (myoblasts) were taken at day 3 after plating. To induce differentiation, the medium was changed at day 4 after plating to DMEM supplemented with 0.25% FBS and 0.25% horse serum. Samples of differentiated cells (referred to as myotubes) were taken 24 h after.

**Muscle.** Regenerating skeletal muscle (extensor digitorum longus muscle) of adult male Wistar rats (age 2 months) were obtained after crush according to Bassaglia and Gautron (1995). The muscle crushing protocol was performed after the rats had been anesthetized with pentobarbital (0.2 ml/100 g). At days 1, 3, and 9 after crush, animals were terminally anesthetized with pentobarbital, and regenerating muscles were removed and weighed. Intact muscles were used as a control. The muscles were frozen in liquid nitrogen and stored at −80°C until use.

**Skin.** Swiss mice were depilated with depilatory cream 2 days before ulcer generation by intradermal injection of

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Table III. Sulfated GAGs in biological extracts

<table>
<thead>
<tr>
<th></th>
<th>Sulfated GAG</th>
<th>HS</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/mg FTa</td>
<td>μg/mg FTa</td>
<td>μg/mg FTa</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td>SD (%)</td>
<td>SD (%)</td>
</tr>
<tr>
<td>1 days after crush</td>
<td>0.14</td>
<td>0.009 (6.43)</td>
<td>0.10</td>
</tr>
<tr>
<td>3 days after crush</td>
<td>0.73</td>
<td>0.066 (9.04)</td>
<td>0.30</td>
</tr>
<tr>
<td>9 days after crush</td>
<td>1.55</td>
<td>0.023 (1.48)</td>
<td>0.93</td>
</tr>
<tr>
<td>Intact muscle</td>
<td>0.45</td>
<td>0.012 (2.66)</td>
<td>0.34</td>
</tr>
<tr>
<td>Skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulcerateb</td>
<td>8.1</td>
<td>0.202 (2.49)</td>
<td>4.42</td>
</tr>
<tr>
<td>Normal</td>
<td>17.6</td>
<td>0.402 (2.28)</td>
<td>8.75</td>
</tr>
</tbody>
</table>

°Ft, fresh tissue.

bUlcerate skin sample was taken at day 13 after doxorubicine injection.
doxorubicin in the back (Balsari et al., 1989; Rudolph et al., 1979). Mouse skin was at its maximum of ulceration 11 days after injection. Healthy or ulcerated skin fragments were then taken on the back of each animal, which were terminally anesthetized with pentobarbital. Samples were stored at -80°C until use.

Housing of animals and anesthesia were performed following the guidelines established by the Institutional Animal Welfare with the European guide for care and use of laboratory animals.

Preparation of biological samples for the DMMB assay
Myogenic cell cultures, skeletal muscles, and skin were used as biological materials. Whatever its origin, material was digested in a solution of 50 µg/ml proteinase K in 100 mM K_2HPO_4 pH 8.0 at 56°C overnight. Proteinase K was then inactivated by heating the preparation 10 min at 90°C (Calabro et al., 2000). After centrifugation, digested tissue was filtered through an Ultrafree filter to eliminate DNA and tissue debris from the extract. This preparation was used for sulfated GAG quantification. The amount of DNA in samples was determined by dianaminophenyl indole (DAPI) assay using salmon sperm DNA as standard (Brunk et al., 1979).

Spectroscopic determination of sulfated GAG: GAG-DMMB complexation/decomplexation
The content of sulfated GAGs was determined using the DMMB solution as follows. 1 ml working DMMB solution was added to 100 µl proteinase K–treated sample, and the mixture was vigorously vortexed for 30 min to promote complete complexation of the GAG with DMMB. The insoluble GAG-DMMB complex was then separated from the soluble materials, including DMMB excess, by centrifugation (12,000 x g, 10 min). The supernatant was discarded, and the pellet was dissolved with the decomplexation solution. The added volume of this solution was adjusted according to the quantity of GAGs. For samples containing GAGs at the microgram level, 1 ml decomplexation solution was added. For lower quantities (<1.0 µg) 500 µl was added. Decomplexation was achieved by shaking the mixture for 30 min. Absorbance was measured at 656 nm. Sulfated GAG quantities in biological samples were determined by comparison with a calibration curve of CS solutions used as standard and treated as described. For submicrogram and microgram quantities of GAGs, calibration curves were used from 0.025 to 1 µg/ml and from 1.0 to 5 µg/ml of CS, respectively. HS was also used as standard, giving similar curves but less than 5% difference.

Quantification of HS
HS was eliminated from the original sample according to Bosworth and Scott (1994) with some modifications. In brief, 100 µl proteinase K–treated sample was mixed with 100 µl sodium nitrite (5%) and 100 µl acetic acid (33%). Samples were gently shaken and kept at room temperature for 1 h. To stop the reaction, 100 µl ammonium sulfamate (12.5%) was added, and the mixture was shaken for a further 5 min. Remaining sulfated GAGs were determined in 100 µl of this nitrous acid reaction mixture by following the DMMB protocol as described. The GAG remaining in the sample represented O-sulfated GAGs, including CS. The N-sulfated GAGs (HS) content was then calculated as the difference between the total GAGs and the O-sulfated GAGs in each sample. A calibration curve was constructed as shown by preparing mixtures of known amounts of CS treated in the same way.

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Abbreviations
CS, chondroitin sulfate; CV, coefficient of variation; DAPI, dianaminophenyl indole; DMEM, Dulbecco modified Eagle medium; DDMB, 1,9-dimethylmethylene blue; FBS, fetal bovine serum; GAG, glycosaminoglycan; GuHCl, guanidine hydrochloride; HS, heparan sulfate.

References


