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# The polarity and magnitude of ambient charge influences three-dimensional neurite extension from DRGs

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**Abstract:** Sulfated proteoglycans have inhibitory effects on neurite extension, and the negative charge of the glycosaminoglycan side chains may be involved in the inhibitory process. The main goal of this study is to investigate the effects of charge on three-dimensional neurite extension. Various concentrations of dermatan sulfate (DS), a chondroitin sulfate glycosaminoglycan, and consequently, various degrees of negative charge were presented on three-dimensional agarose hydrogels and the effect of charge on neurite extension from primary neurons was investigated. Dose-response experiments were also performed with the polycationic (positively charged) polysaccharide chitosan covalently coupled to agarose. The amount of DS or chitosan coupled to the agarose gel was quantified via metachromatic dye or

Fourier transform infrared spectroscopy methods, respectively. The length of embryonic day 9 (E9) chick dorsal root ganglia neurites extended through charged agarose gels is dependent on the polarity and quantity of ambient charge. The inhibitory effects of the sulfated DS and the enhancing effects of the polycationic chitosan on neurite extension decrease as the amount of DS or chitosan coupled to agarose is decreased. These findings indicate that primary neural process extension is influenced by the polarity of ambient charge in a dose-responsive manner. © 2000 John Wiley & Sons, Inc. *J Biomed Mater Res*, 51, 510–519, 2000.

**Key words:** agarose; nerve regeneration; chitosan; chondroitin sulfate; glial scar

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## INTRODUCTION

During fetal development, charged molecules such as chondroitin sulfate proteoglycans (CSPG) repel axons and inhibit neural cell adhesion.<sup>1,2</sup> The shift in ratio of growth-inhibiting to growth-promoting molecules in the developing mammalian retina may help control the onset of ganglion cell differentiation and initial direction of axon growth.<sup>3</sup> Also, CSPGs are re-expressed within the gray and white matter of the central nervous system (CNS) in adult animals after injury.<sup>4–6</sup> This expression of CSPG has been correlated with inhibited neurite outgrowth on glial scars *in vitro* and *in vivo*, suggesting that the relative balance of enhancing versus inhibitory factors in the local environment following CNS injury may influence the extent of regeneration.<sup>4–6</sup> When bound to growth-promoting fibronectin substrates, CSPGs inhibit chick dorsal root

ganglion (DRG) neurite extension in a concentration dependent manner.<sup>7</sup>

Many mechanisms have been proposed to describe the inhibitory functions of sulfated proteoglycans. One proposed mechanism maintains that the negative charge of the glycosaminoglycan (GAG) side chains inhibits neurite outgrowth.<sup>1</sup> The variations in the inhibitory effect between proteoglycans may be due to differences in the length and/or conformation of the GAG chains or differences in the degree or pattern of sulfation (and consequently negative charge) of the GAG chains,<sup>1,2</sup> or their ability to localize growth factors.<sup>8,9</sup>

In two-dimensional (2D) experiments on flat tissue culture substrates, it is possible to separately investigate the roles of charge and chemistry as demonstrated by Valentini et al.<sup>10,11</sup> using electret films. However, the influence of biologically based electrical charge on three-dimensional (3D) neural process extension remains unclear. Because it is known that the differentiated phenotype of cells *in vitro* is regulated by both the biochemical composition and mechanical properties of the substrate, 3D biomaterials may provide a more relevant emulation of the *in vivo* extracellular matrix than their 2D counterparts. The main goal of this study is to investigate the effects of ambient

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charge in a 3D hydrogel in a manner relevant to both boundary formation during development, and glial scar tissue formation associated with the failure of nerve regeneration *in vivo*. We hypothesize that 3D hydrogels may be used to simulate conditions prevalent during development and may potentially serve as 3D “bridge materials” to encourage regeneration across nerve gaps in the peripheral nervous system and CNS.

Charge neutral agarose gels have been used previously for 3D culture of neural cells *in vitro*<sup>12–14</sup> and as a matrix filling polymer guidance channels *in vivo*.<sup>15</sup> In the current study, we systemically and quantitatively investigate the influence of charge associated with the sulfated GAG side chains of CSPG on the inhibition of primary neural process extension, using various concentrations of a chondroitin sulfate GAG and consequently, various degrees of charge in 3D agarose matrices being developed in our laboratory. To clarify the influence of polarity of charge on neurite extension, dose-response experiments were also performed with the polycationic polysaccharide chitosan covalently coupled to agarose gel.

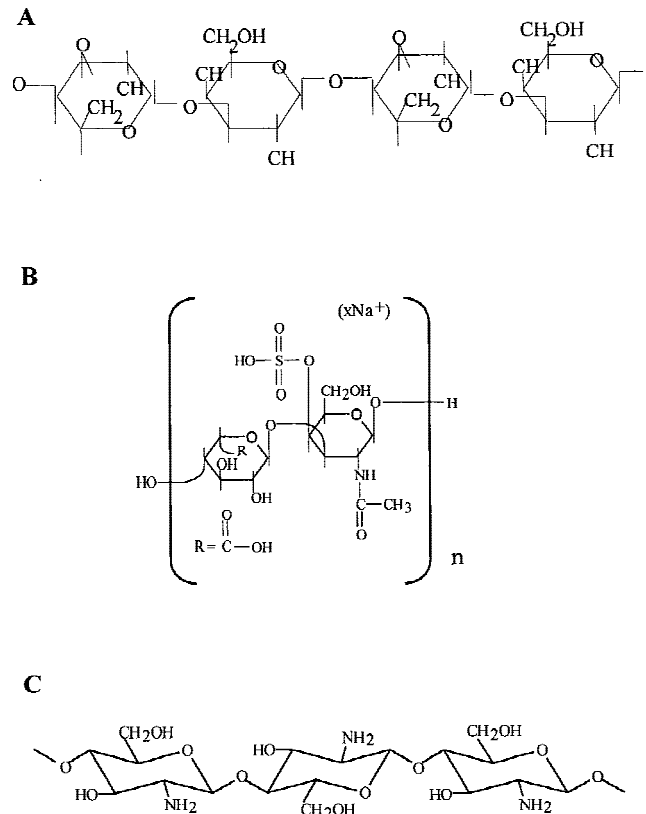
## MATERIALS AND METHODS

### Agarose gel preparation

Agarose is a clear, thermoreversible hydrogel composed of alternating copolymers of 1,4-linked 3,6-anhydro- $\alpha$ -L-galactose and 1,3-linked  $\beta$ -D-galactose polysaccharides derived from red algae [see structure in Fig. 1(A)]. The mechanism for gelation of agarose involves a shift from a random coil of the polysaccharide in solution to an intermediate double helix form in early gelation ultimately to bundles of double helices in final gelation.<sup>16,17</sup> Water soluble SeaPrep<sup>®</sup> agarose (FMC Bioproducts, Rockland, ME) used in this study, is a hydroxyethylated agarose that gels at 17°C and once gelled, remains stable unless reliquified by heating to 50°C. When used for cell culture, the agarose was dissolved in pH 7.4 phosphate buffered saline (PBS) (Dulbecco's phosphate buffered saline; GIBCO, Grand Island, NY) and filtered through a 0.45  $\mu$ m syringe filter (Nalgene<sup>®</sup> syringe filters; Nalge Co., Rochester, NY). SeaPrep agarose gels will be referred to as “unmodified” agarose gel.

### Introduction of charged biopolymers into 3D agarose scaffolds

Polyanionic chondroitin sulfate type B and polycationic chitosan were the “biopolymers” used to introduce ambient charge into agarose gels by covalent coupling [see Fig. 1(B) and (C) for structures]. Chondroitin sulfate is the sulfated (therefore negatively charged) GAG side chain portion of chondroitin sulfate proteoglycan. Chondroitin sulfate is a



**Figure 1.** Schematics of (A) SeaPrep agarose, a hydroxyethylated agarose with alternating copolymers of 1,4-linked 3,6-anhydro- $\alpha$ -L-galactose and 1,3-linked  $\beta$ -D-galactose polysaccharides, and charged biopolymers, (B) chondroitin sulfate B (Dermatan sulfate) of the form copoly( $\beta$ -iduronic acid-[1 $\rightarrow$ 3]- $N$ -acetyl- $\beta$ -galactosamine-4-sulfate-[1 $\rightarrow$ 4]) chondroitin sulfate, and (C) chitosan, PROTOSAN CL 210, a linear cationic polysaccharide consisting of  $\beta$ -(1-4) linked 2-acetamido-2-deoxy- $\beta$ -D-glucopyranose and 2-amino-2-deoxy- $\beta$ -D-glucopyranose.

linear polymer of repeating disaccharides of various lengths, degrees of sulfation and epimerization.<sup>18</sup> The chondroitin sulfate GAG used in this study, chondroitin sulfate type B also known as dermatan sulfate (DS) (MW: 14 kDa, dermatan sulfate from porcine intestinal mucosa; Sigma Chemical Co., St. Louis, MO) was of the form copoly( $\beta$ -iduronic acid-[1 $\rightarrow$ 3]- $N$ -acetyl- $\beta$ -galactosamine-4-sulfate-[1 $\rightarrow$ 4]). This particular GAG was chosen based on several studies citing an injury-induced increase of dermatan sulfate proteoglycan (DSPG), along with other sulfated proteoglycans, produced by astrocytes *in vitro*.<sup>19,20</sup> Lesion-induced up-regulation of the chondroitin/dermatan sulfate proteoglycans (CS/DSPGs), biglycan, and decorinin, has been detected in the postcommissural fornix of the adult rat.<sup>21,22</sup> Also, unpublished experiments performed in our laboratory indicated that DS inhibited neurite extension from embryonic day 9 (E9) chick DRGs more effectively than other sulfated GAGs.

Chitosan is a linear cationic polysaccharide consisting of  $\beta$ -(1-4) linked 2-acetamido-2-deoxy- $\beta$ -D-glucopyranose and 2-amino-2-deoxy- $\beta$ -D-glucopyranose. The degree of deacetylation influences both the structure and the physical properties of chitosan.<sup>23,24</sup> The chitosan used in this study,

PROTOSAN™ CL 210 (MW: 130–160 kDa; Pronova Biopolymer, Oslo, Norway), has approximately 0.85 degree of deacetylation and is soluble in water. The chitosan was the generous gift of Dr. Øyvind Skaugrud, Pronova Biopolymer, Oslo, Norway. The polycationic (as a result of amine groups) chitosan was chosen based on studies indicating increased cell attachment when using chitosan as a component of a 2D fibroblast culture substrate.<sup>25,26</sup> Additionally, a chitosan matrix has been used successfully in the lumen of polyacrylonitrile/polyvinylchloride (PAN/PVC) microcapsules for the support of fibroblasts and PC12 cell neurite extension.<sup>27</sup>

### Covalent coupling of charged biopolymers to agarose hydrogels

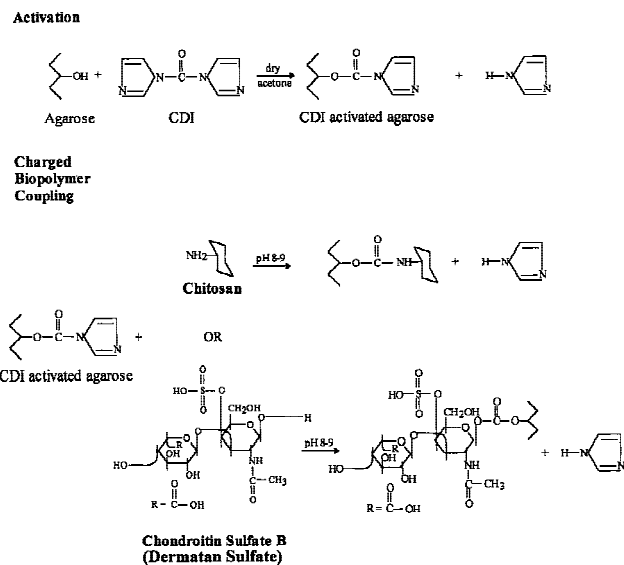
Four different concentrations of the polyanionic polysaccharide dermatan sulfate and the polycationic polysaccharide chitosan were covalently coupled to the hydroxyl backbone of agarose in various concentrations to facilitate the investigation of the influence of degree of charge on 3D neurite extension (Table I). Agarose gels were derivatized with 1,1'-carbonyldiimidazole (CDI) (Aldrich Chemical Co., Inc., Milwaukee, WI) using a modification of the protocol described by Hern<sup>28</sup> and Bellamkonda et al.<sup>12</sup> (see Fig. 2 for schematic). Briefly, an 8-mL gel sample of 1.5% agarose was dehydrated by repeated washes in increasing concentrations of acetone followed by dry acetone (dried under 4 Å molecular sieves; Sigma), and cut into chunks as small as possible. Five milliliters of a CDI solution prepared in dry acetone (30 mg/mL) was added to the acetone-washed agarose gel. The activation reaction was allowed to proceed for 1 h with gentle agitation. The gel was then washed with dry acetone three times while being shaken vigorously for 5 min per wash to remove unbound CDI. The activated gel was then rehydrated in 6 mL of 100 mM sodium bicarbonate buffer solution (pH 8.5). A certain concentration of either DS or chitosan was dissolved in deionized water and the solution added to the activated gel (see Table I for exact concentrations). The coupling reaction was allowed to proceed overnight under gentle agitation. The DS- or chitosan-coupled agarose solution was gelled at 4°C. The modified

**TABLE I**  
Amount of DS or Chitosan Added to the CDI-Activated Agarose During the Coupling Reactions

Modified Agarose "Name"	Biopolymer Added	Mass Added (mg) <sup>a</sup> [mg/mL Agarose] <sup>b</sup>
DS10	Dermatan sulfate	10 [0.83]
DS20	Dermatan sulfate	20 [1.67]
DS30	Dermatan sulfate	30 [2.50]
DS40	Dermatan sulfate	40 [3.33]
Chit30	Chitosan	30 [2.50]
Chit60	Chitosan	60 [5.00]
Chit90	Chitosan	90 [7.50]
Chit120	Chitosan	120 [10.00]

<sup>a</sup>Note amount of biopolymers added into 8 mL of 1.5% agarose gels.

<sup>b</sup>The amount of biopolymers added into 1 mL of 1.0% agarose gels.



**Figure 2.** Schematic of 1,1'-carbonyldiimidazole coupling chemistry used to link the charged biopolymers, chitosan and dermatan sulfate (DS, chondroitin sulfate B), to agarose gels.

gel was quenched in the sodium bicarbonate buffer overnight to hydrolyze unreacted CDI groups and washed thoroughly with deionized water, lyophilized, and reconstituted in PBS to the desired gel concentration of 1.0% w/v. Agarose gels covalently coupled with either DS or chitosan will be referred to as “coupled” or “modified” agarose gels.

### Analytical verification of coupling chemistry/quantification of coupled charged biopolymer

#### Quantification of coupled DS by dimethyl-methylene blue

It has been demonstrated previously that 1,9-dimethyl-methylene blue (DMMB) is a strong metachromatic dye for the detection of sulfated glycosaminoglycans.<sup>29,30</sup> A standard curve for DS was established using a modification of the methods of Farndale et al.<sup>29</sup> Briefly, 2.5 mL of the DMMB (Aldrich) color reagent was mixed carefully with 100  $\mu$ L of known concentrations of DS dissolved in liquid 1.0% Sea-Prep agarose at room temperature. For all tests, the baseline consisted of 2.5 mL of the DMMB color reagent mixed with 100  $\mu$ L of 1.0% Sea-Prep agarose. A 100  $\mu$ L sample of each 1.0% w/v DS-coupled agarose gel was tested. Fifteen seconds after gently mixing the color reagent and the DS-coupled agarose gel, the absorbance at 525 nm was measured by spectrophotometer (Spectronic® Genesys™ 2, Milton Roy Co., Rochester, NY).

#### Fourier transform infrared (FTIR) quantification of coupled chitosan

Infrared spectroscopy was used to verify the coupling of chitosan to agarose. After dehydration by lyophilization to

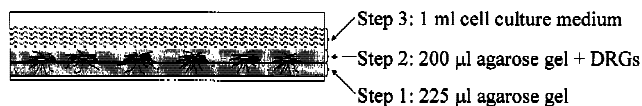
minimize —OH interference, 9 mg of each of the modified agarose gel samples was thoroughly ground with 100 mg KBr in powder form. The resulting mixture was pressed into films and analyzed by FTIR spectrometer (Biorad FTS-575C; BioRad Laboratories, Cambridge, MA). In addition to the modified gels, pure agarose and pure chitosan samples were analyzed as controls. Peaks unique to agarose and chitosan were identified from the FTIR spectra of these “pure” samples. This unique peak will be referred to as the “characteristic peak.” According to Beer’s law, the ratio of the absorbance values of the characteristic peaks of chitosan and agarose provided insight into the relative amount of chitosan coupled in each of the modified agarose gels.

### Culture of embryonic chick dorsal root ganglia in various agarose gel formulations

SeaPrep agarose is capable of supporting neurite extension from E9 DRGs and 1.0% SeaPrep has the optimal physical structure for E9 DRG neurite extension compared with the other agarose gel concentrations.<sup>14</sup> Dorsal root ganglia were dissected from E9 chick embryos and cultured in 1.0% w/v unmodified agarose gel and each of the 1.0% modified agarose gels using previously published protocols (see Fig. 3 for schematic).<sup>12,14</sup> Briefly, the bottom of one well of a 24-well tissue culture dish (Costar Corp., Cambridge, MA) was coated with 225  $\mu$ L of the 1.0% agarose solution at 37°C. The dish was then placed at 4°C for approximately 15 min to allow the agarose to gel. Typically, 4 DRGs were added to another 200  $\mu$ L of the agarose solution (to a final concentration of 1.0%) in a 1 mL syringe at 37°C. The liquid agarose solution and the DRGs were carefully mixed by manually pumping a syringe plunger. The entire contents of the syringe were added to the well containing the previously gelled agarose. The dishes were then placed at 4°C and the solution gelled, trapping the DRGs in the 3D scaffold. One milliliter of Dulbecco’s modified eagle’s medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 1% penicillin-streptomycin (GIBCO) and 50 ng/mL of mouse nerve growth factor (mNGF) (2.5 S, MW: ~26 kDa; Alomone Labs, Jerusalem) was added to the top of the gels and the dishes stored at 37°C in 5% CO<sub>2</sub> and 95% humidity. The cell-culture medium was changed every 48 h.

### Analysis and quantification of dorsal root ganglia neurite extension

*In vitro* analysis and quantification of neurite extension in the unmodified and modified agarose gel scaffolds were



**Figure 3.** Schematic of typical 1.0% (w/v) agarose gel culture of E9 chick DRGs described in the text. Represented in cross-section (not to scale) is one well of a 24-well tissue culture dish.

performed after 4 days of culture using a digital image analysis system consisting of a Nikon Eclipse TE 300 inverted microscope (Nikon Corp., Tokyo, Japan), a monochrome CCD camera (Javelin JE7862; Javelin Electronics, Japan), and a Power Macintosh® (Apple Computer, Inc., Cupertino, CA) equipped with the LG-3-PCI frame grabber (Scion Corp., Frederick, MD) and running the NIH Image software package.<sup>31</sup> Light microscope images of a DRG in 3D culture were captured and the eight longest neurites were traced and their length quantified using NIH Image. This process was repeated for at least four DRGs before the “maximal neurite length” for the sample was calculated by averaging the eight measured neurite lengths from each DRG. The measured neurite lengths were normalized with respect to the neurite length in 1.0% w/v unmodified agarose control common to all experiments conducted in this study. Each experiment was conducted at least twice and the results were pooled. A minimum of eight neurites from each of at least eight separate DRGs were measured in each agarose gel type. Two-tailed Student *t* tests were performed and a *p* value less than 0.05 was considered to be statistically significant.

### Porosity analysis of DS and chitosan-modified agarose gels

To evaluate the mechanical properties of the modified agarose gels, the porosity of the DS and Chitosan-modified agarose gels (1.0%, w/v) was analyzed by measuring their pore radius with hydraulic permeability testing.<sup>14</sup>

### Specificity of DS inhibitory effect on neurite extension

Five units of chondroitinase ABC (Sigma) was added to 4 mL of 1.0% (w/v) DS-coupled agarose gel in PBS solution (pH = 7.4) at 37°C for 3 h. After boiling for 1 min, the solution was then put in 4°C for 15 min to form the gel. The small molecules formed and extra chondroitinase ABC was removed by washing extensively with PBS. E9 DRGs were cultured three-dimensionally in chondroitinase ABC treated 1.0% (w/v) DS-coupled agarose gel as described above, and the DRG neurite extension was evaluated.

## RESULTS

### Quantification of coupled DS by dimethyl-methylene blue

The amount of DS covalently coupled in the DS-coupled agarose gels was calculated by comparing the absorbance of the DS-coupled agarose gels with the DS standard curve. The DS-coupled agarose gel samples were diluted so that the absorbances fell in

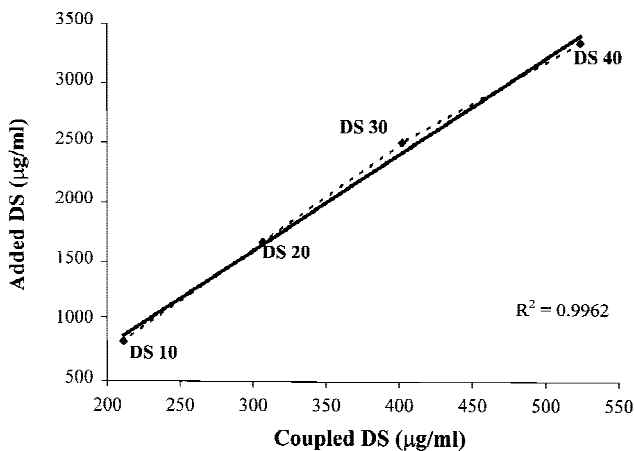
the linear region of the standard curve. As seen in Figure 4, the amount of DS coupled to the agarose gel during the modification process increased linearly ( $R^2 = 0.9962$ ) as the amount of DS added during the coupling process was increased.

### Identification of characteristic peaks/verification of chitosan coupling

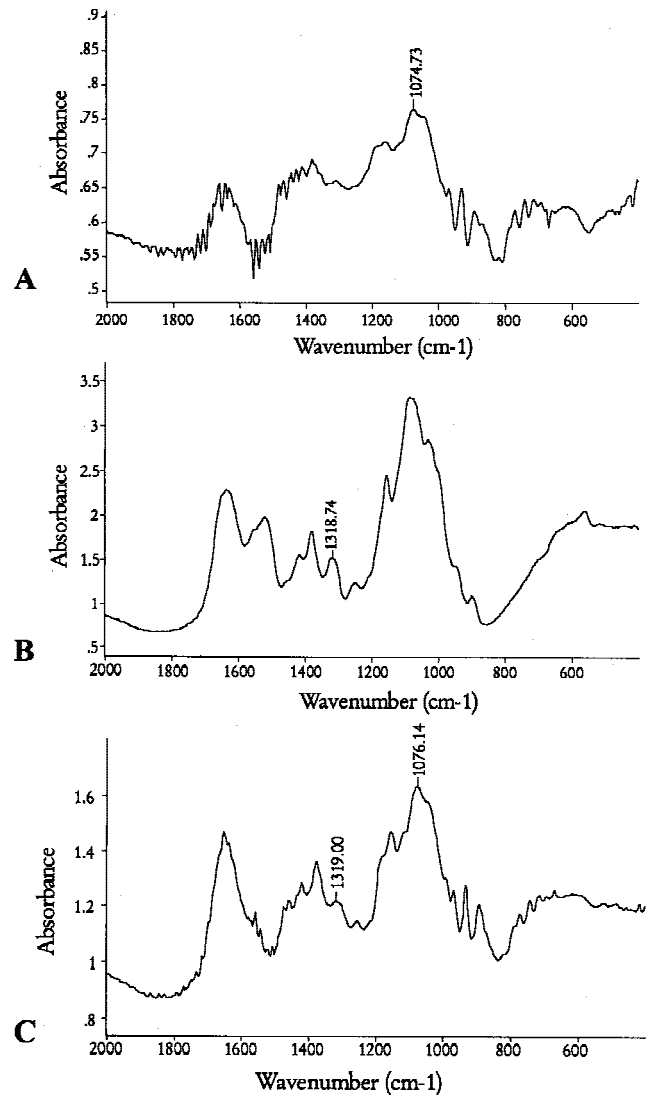
The agarose spectrum [Fig. 5(A)] presented peaks near  $1074\text{ cm}^{-1}$  due to primary ( $-\text{CH}_2-\text{OH}$ ) and secondary ( $-\text{CH}-\text{OH}$ ) alcohol groups.<sup>32,33</sup> Although these groups are also present in the chitosan structure, the  $1074\text{ cm}^{-1}$  peak is not present in the chitosan FTIR spectrum and was therefore assumed to be characteristic of agarose. The chitosan spectrum [Fig. 5(B)] exhibited a peak near  $1318\text{ cm}^{-1}$  typical of primary amines ( $-\text{NH}_2$ ) and secondary amines ( $-\text{NH}-\text{R}$ ), both of which are present only in the structure of chitosan.<sup>33</sup> The presence of these peaks in the FTIR spectra of the modified agarose gels confirms the successful covalent coupling of chitosan to agarose.

### Quantification of coupled chitosan by FTIR spectroscopy

The relative amount of chitosan covalently coupled in the chitosan-modified agarose gels was calculated by comparing the ratios of the chitosan absorbances (at the characteristic peak, near  $1318\text{ cm}^{-1}$ ) with the agarose absorbances (at the characteristic peak, near  $1076\text{ cm}^{-1}$ ) in the four chitosan-coupled FTIR spectra [for example, the Chit120 spectrum is presented in Fig. 5(C)]. According to this analysis, the amount of chito-



**Figure 4.** Relationship of amount of DS added to the CDI-activated agarose gel to the amount of covalently coupled DS detected by DMMB. The solid line is a linear fit ( $R^2 = 0.9962$ ) of the data.



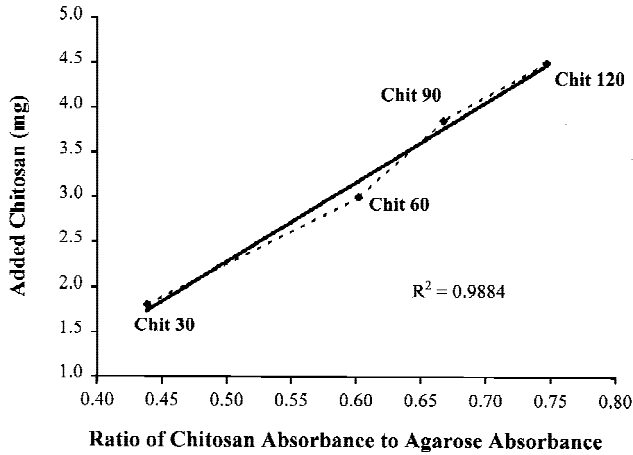
**Figure 5.** FTIR spectra of (A) SeaPrep agarose, (B) chitosan, and (C) Chit120, a chitosan-coupled agarose gel. The characteristic peaks are indicated with their specific wavenumber.

san actually coupled to the agarose gel during the modification process increased linearly ( $R^2 = 0.9884$ ) as the amount of chitosan added during the coupling process was increased (Fig. 6).

### DRG neurite extension in charged biopolymer-coupled agarose gels

#### Influence of polarity of charge on neurite extension in 3D matrix

Neurite extension from E9 DRGs was quantified on the fourth day of culture. Figure 7 illustrates the effect of ambient charge polarity on neurite extension through modified agarose hydrogels. The data repre-

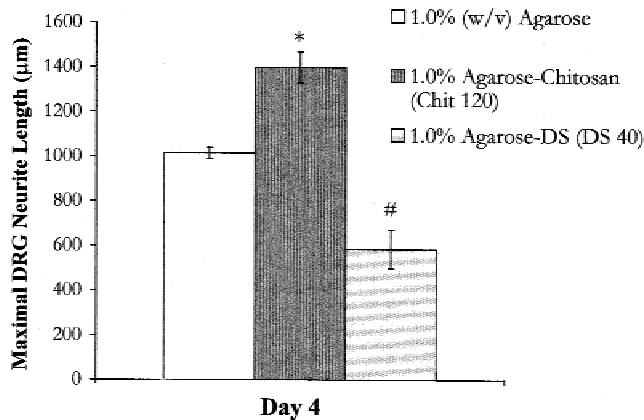


**Figure 6.** The amount of chitosan added to the CDI-activated agarose gel compared with the ratio of chitosan absorbance to agarose absorbance. The solid line is a linear fit ( $R^2 = 0.9884$ ) of the data.

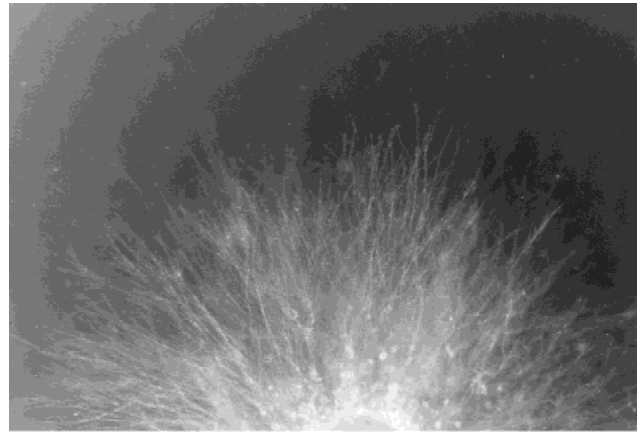
sent the highest concentrations of each biopolymer coupled to the CDI-activated agarose. The DS-coupled agarose gel (DS40) inhibited neurite extension by 42% ( $p < 0.001$ ) compared with the 1.0% unmodified agarose control. On the other hand, the chitosan-coupled agarose gel (Chit120) enhanced neurite extension by 38% ( $p < 0.001$ ). Images of neurites extending through unmodified, DS-coupled, and chitosan-coupled agarose gels can be seen in Figure 8.

Influence of quantity of charge on neurite extension

The amount of DS added to the CDI-activated agarose gel determined the inhibition observed after 4



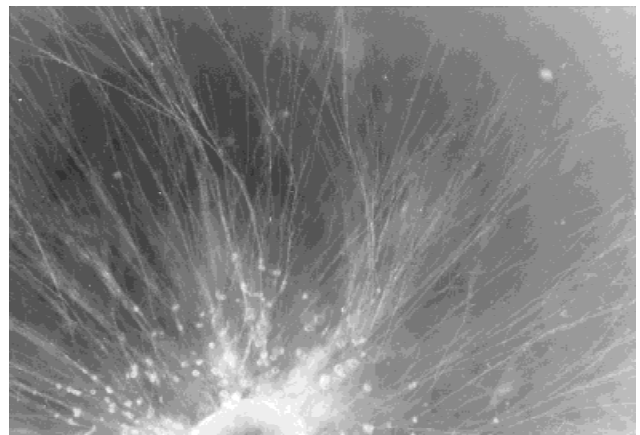
**Figure 7.** Influence of polarity of charge on E9 chick DRG neurite extension through modified agarose hydrogels at 4 days of culture (mean  $\pm$  SEM). \* indicates statistical significance ( $p < 0.001$ ) with respect to the 1.0% SeaPrep agarose control. # indicates statistical significance with respect to the 1.0% SeaPrep agarose control ( $p < 0.001$ ) and Chit120 ( $p < 0.001$ ).



(A)



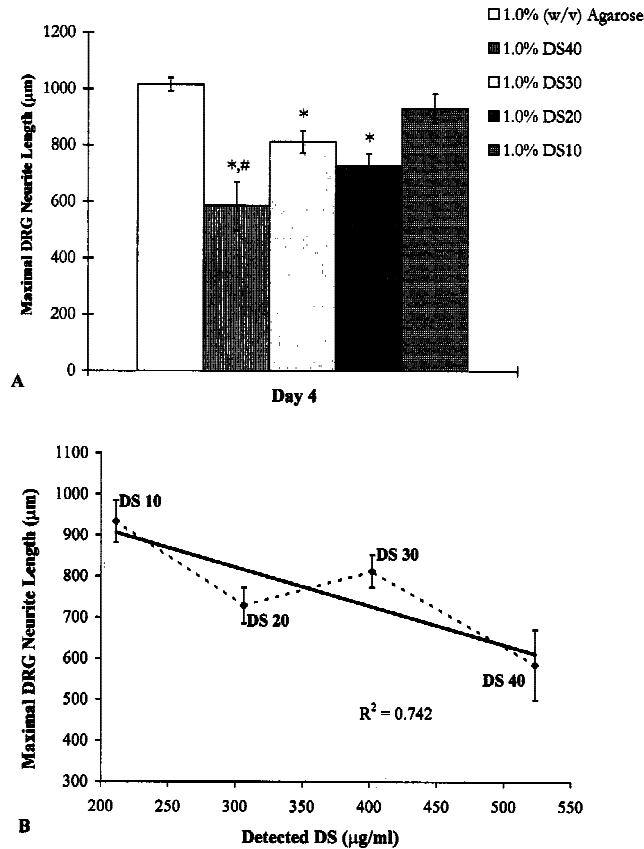
(B)



(C)

**Figure 8.** Photomicrographs of E9 chick DRGs extending neurites in unmodified, DS-coupled, and chitosan-coupled agarose gels after 4 days in culture. (A) 1.0% w/v agarose control (original magnification  $\times 80$ ); (B) 1.0% DS40 (original magnification  $\times 80$ ); and (C) 1.0% chit120 (original magnification  $\times 80$ ).

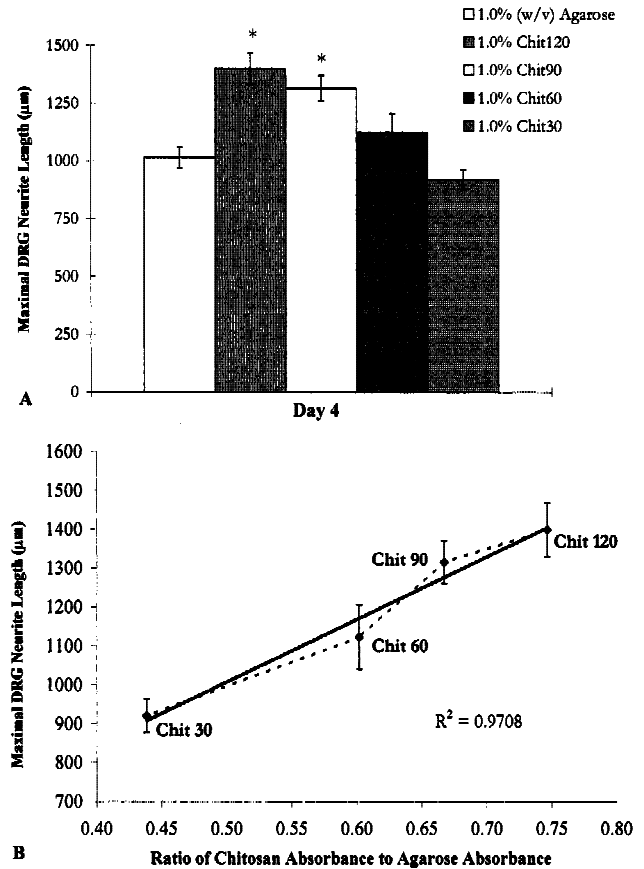
days of culture [Fig. 9(A)]. That is, the more DS that was coupled to the CDI-activated agarose gel, the greater the inhibition of neurite extension [Fig. 9(B)]. DS40 inhibited neurite extension by 42% ( $p < 0.001$ ) compared with control, and significantly inhibited ex-



**Figure 9.** Influence of negative charge quantity on neurite extension through modified agarose hydrogels after 4 days of culture. (A) Histogram comparing E9 chick DRG neurite extension in DS-coupled and unmodified agarose gels (mean  $\pm$  SEM). \* indicates statistical significance is with respect to the unmodified 1.0% w/v agarose control. # indicates statistical significance is with respect to DS30. (B) Influence of amount of DS coupled to the agarose gel on neurite extension through modified agarose hydrogels (mean  $\pm$  SEM). The solid line is a linear fit ( $R^2 = 0.742$ ) of the data.

tension compared with DS30 ( $p < 0.05$ ) and DS10 ( $p < 0.005$ ). DS30 and DS20 also significantly inhibited neurite extension compared with control by 20% ( $p < 0.001$ ) and 28% ( $p < 0.001$ ), respectively. The difference in average maximal neurite extension between DS20 and DS30 is not statistically significant. Although not statistically significant, DS10 inhibited neurite extension by 8% compared with control.

Similarly, the neurites extended through the various chitosan-coupled agarose gels responded in a chitosan concentration dependent manner (Fig. 10). The modified gel, Chit120 supported 38% ( $p < 0.001$ ) longer neurites than the 1.0% unmodified agarose control and significantly longer neurites than Chit30 ( $p < 0.001$ ). As the concentration of chitosan decreased, the neurite extension decreased linearly ( $R^2 = 0.9708$ ). Chit90 supported a 30% ( $p < 0.001$ ) increase in neurite length over control and statistically significantly increased neurite extension over Chit30 ( $p < 0.001$ ), whereas Chit60 supported an 11% increase over control.



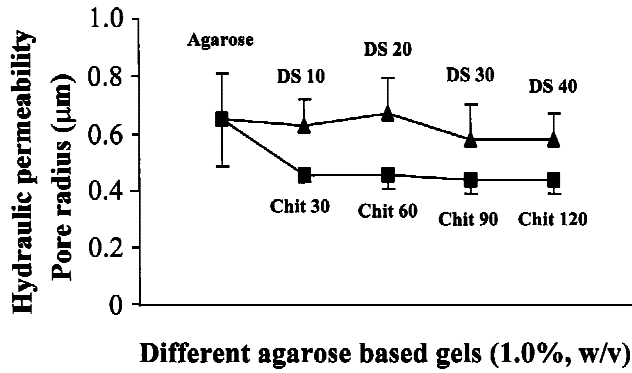
**Figure 10.** Influence of positive charge quantity on neurite extension through modified agarose hydrogels after 4 days of culture. (A) Histogram comparing E9 chick DRG neurite extension in chitosan-coupled and unmodified agarose gels (mean  $\pm$  SEM). \* indicates statistical significance is with respect to the unmodified 1.0% w/v agarose control. (B) Influence of amount of chitosan coupled to the agarose gel on neurite extension through modified agarose hydrogels (mean  $\pm$  SEM). The solid line is a linear fit ( $R^2 = 0.9708$ ) of the data.

### Porosity analysis of DS and chitosan-modified agarose gels

The porosity measurements showed that the pore radius of DS-modified gels did not have significant difference with that of unmodified agarose gel, and the pore sizes of Chitosan-modified gels decreased compared with unmodified agarose gel (Fig. 11).

### Specificity assay for DS inhibitory effect on neurite extension

The DS-coupled agarose gel (DS40) inhibited neurite extension by 42% ( $p < 0.001$ ). After treatment of DS-coupled agarose gel with chondroitinase ABC, the DRG neurite length was significantly higher ( $p < 0.001$ ) than that in the untreated DS-coupled agarose

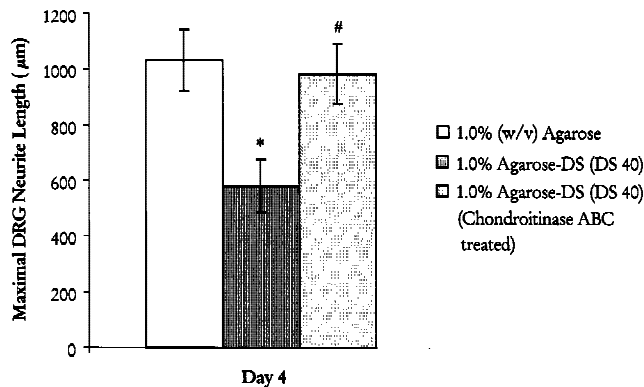


**Figure 11.** Pore radius of different agarose-based gels calculated by hydraulic permeability.

gel (Fig. 12). There was no significant difference for DRG neurite length in 1.0% (w/v) chondroitinase ABC-treated DS-coupled agarose gel and in 1.0% (w/v) unmodified agarose gel.

**DISCUSSION**

Previous studies have demonstrated that agarose hydrogels have the necessary physiochemical structure to support 3D neurite extension from anchorage-dependent primary neural cells including E9 chick dorsal root ganglia.<sup>12-14</sup> Because primary chick dorsal root ganglia have no known receptors for agarose, neural cell response to changes in the ambient charge of the agarose gel scaffold is able to be investigated. In this study, the relationship between the polarity of ambient charge and neurite extension from primary sensory ganglia (DRGs) was investigated. Also, the influence of the quantity of charge incorporated into



**Figure 12.** Influence of chondroitinase ABC treatment on DRG neurite extension in DS-coupled gels at 4 days of culture (mean  $\pm$  SEM). \* indicates statistical significance ( $p < 0.001$ ) with respect to 1.0% SeaPrep agarose control. # indicates statistical significance with respect to the DS-coupled agarose gel (DS40).

the 3D environment on neurite extension was determined in this dose-response study.

A slightly modified version of the DMMB sulfated GAG detection technique described by Farndale et al.,<sup>29</sup> was effective in quantifying the amount of covalently coupled DS present in each of the modified agarose gels (DS40, DS30, DS20, and DS10). The range of concentrations of coupled DS (Fig. 6; approximately 200–525  $\mu\text{g}/\text{mL}$ ) is similar to the range of GAG concentrations tested in other investigations of the effect of GAGs on neurite extension.<sup>7</sup>

Chitosan was successfully covalently coupled to agarose gel as confirmed by FTIR spectroscopy. The maximal amount of chitosan added to the activated agarose gel (see Table I) was determined by the limited solubility of chitosan at the pH required for the CDI coupling chemistry. The relative amounts of chitosan coupled to each modified agarose gel (i.e., Chit120, Chit90, Chit60, and Chit30) was also determined by FTIR spectroscopy.

The results of the DMMB and FTIR quantification confirm the efficacy of the CDI coupling chemistry. Even at the highest concentrations of DS and chitosan tested in this study, no limit of coupled biopolymer was reached. Therefore, within the range of concentrations tested, the coupling reaction is limited by the amount of the biopolymer added to the CDI-activated agarose gel.

In this study, we show that the length of E9 chick DRG neurites extended through modified agarose gels is dependent on the polarity and quantity of coupled charged biopolymer. The inhibitory effects of the sulfated DS and the neurite extension promoting effects of the polycationic chitosan decrease as the amount of biopolymer coupled to agarose is decreased. The maximally coupled polyanionic modified agarose gel (i.e., the negatively charged DS40) maximally inhibited the neurite extension, whereas the maximally coupled polycationic modified agarose gel (i.e., the positively charged Chit120) permitted the most enhanced neurite extension. These findings corroborate our earlier findings<sup>14</sup> that a negative ambient charge inhibits whereas a positive ambient charge enhances E9 chick DRG 3D neurite extension, and shows that there exists a neuronal dose response to degrees of ambient charge. The inhibitory and enhancing effect on neurite extension is attributed to the charge of the coupled DS or chitosan, respectively.

These results may have relevance in the basic understanding of boundary formation during development of the nervous system<sup>5</sup> and in the design of techniques to overcome the glial scar. In response to CNS injury, hypertrophied astrocytes and their associated matrices form a glial scar.<sup>6,34</sup> Historically, the physical characteristics of the glial scar have been hypothesized as the barrier to nerve regeneration. More recently, the idea of the glial scar as a negatively charged inhibitory

environment rather than a purely mechanical barrier to nerve regeneration in the CNS has been gaining acceptance.<sup>34,35</sup> One type of putative inhibitory element of the extracellular matrix is sulfated proteoglycans (including dermatan sulfate)<sup>19,20,36</sup> which have been shown to be produced by astrocytes *in vitro*.<sup>37</sup> As mentioned previously, lesion-induced up-regulation of the CS/DSPGs, biglycan, and decorin, has been detected in the postcommissural fornix of the adult rat.<sup>21,22</sup>

The results for the porosity measurements of DS and chitosan-modified agarose gels demonstrated that the changes in the physical features of agarose gel due to coupling protocols are not responsible for the observed differences in neurite extension both for DS- and chitosan-coupled gels.<sup>14</sup> Therefore, in this study, the DRG neurite extension was indeed influenced by the polarity and magnitude of charge, not by changes in the structure due to coupling. It is also important to note that 50 ng/mL of NGF was present in the culture medium in all of the cultures and the DRGs did not extend neurites in any of our gels without NGF.

After treatment with chondroitinase ABC, the large DS molecule was digested into small molecules, and could be washed away from the gels. DMMB dye assay showed that negative charged sulfate group was removed (data not shown). This explains the loss of the inhibitory effects of DS-coupled agarose gel on DRG neurite extension after chondroitinase ABC treatment. These results indicated that the inhibitory effect of DS-coupled agarose gel for DRG neurite extension was specifically attributed to DS that bound onto the agarose.

The results of this study add strength to the proposed mechanism that implicates the charge associated with the sulfated proteoglycans' GAG side chains as the regulator of neurite outgrowth.<sup>1,2,38</sup> The variations in the inhibitory effect between proteoglycans may be attributed to differences in the length and/or conformation of the GAG chains (as indicated by the GAG concentration dose-response found in this investigation) or differences in the degree or pattern of sulfation of the GAG chains.<sup>1,2</sup> Additionally, the GAG charge influence may be specific for various cell types.<sup>39</sup>

These experiments, in conjunction with our ongoing investigation of 3D physical interfaces, have the potential to enable us to create a model of CS rich boundaries *in vitro*. Once established, this model will be extremely valuable to test strategies such as trophic factor gradients which may help overcome the inhibitory effects of CS rich boundaries in a manner relevant to nerve regeneration.

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