

# Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures

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## Abstract

The optimization of scaffold mechanical properties for neurite extension is critical for neural tissue engineering. Agarose hydrogels can be used to stimulate and maintain three-dimensional neurite extension from primary sensory ganglia in vitro. The present study explores the structure–function relationship between dorsal root ganglion (DRG) neurite extension and agarose gel mechanical properties. A range of agarose gels of differing concentrations were generated and the corresponding rate of E9 DRG neurite extension was measured. Rate of neurite extension was inversely correlated to the mechanical stiffness of agarose gels in the range of 0.75–2.00% (wt/vol) gel concentrations. In addition, we postulate a physical model that predicts the rate of neurite extension in agarose gels, if gel stiffness is a known parameter. This model is based on Heidemann and Buxbaum’s model of neurite extension. These results, if extended to scaffolds of other morphological and chemical features, would contribute significantly to the design criteria of three-dimensional scaffolds for neural tissue engineering. © 2001 Elsevier Science Ltd. All rights reserved.

*Keywords:* Tissue engineering; Scaffolds; Nerve regeneration; Mathematical model

## 1. Introduction

Three-dimensional matrices are significant for peripheral and central nervous system regeneration as potential 3D bridge materials [1–5]. Optimization of matrices that would potentially serve as ideal scaffolds for nerve regeneration will require a better understanding of the effect of substrate mechanical properties on neurite extension. Previously, we have correlated the role of agarose gel pore radius to the tendency of the gel to allow neurite extension and demonstrated its suitability as a 3D substrate for neurite extension [6]. In the present study, we investigate the role of hydrogel mechanical stiffness in neurite extension and suggest that three-dimensional scaffolds for nerve regeneration need to be optimized with respect to their mechanical properties to maximize neurite extension. In this study, we also discuss a mathematical model that predicts the rate of neurite extension based on the stiffness of agarose gels.

The precise mechanism by which mechanical barriers inhibit neurite extension is yet to be elucidated. A structure–function relationship between the mechanical stiffness of the hydrogel and its ability to initiate and maintain maximal neurite extension would be very useful in designing materials with optimal scaffold properties. We propose that the tension-mediated ‘pull’ model of axon elongation proposed by Heidemann and Buxbaum provides a theoretical framework to probe this phenomenon [7]. According to the Heidemann and Buxbaum model, axon initiation occurs when the amoeboid movements along the cell surface generate a putative ‘growth cone’ that exerts a tensional force on the cell membrane that is greater than a threshold value. Heidemann and Buxbaum’s ‘tension-pull’ model of neurite extension and their experimental data in combination with Hooke’s law provide us with a theoretical framework for predicting the rate of neurite extension in 3D agarose gels. We show here that our model can successfully predict the rate of neurite extension in gels of various concentrations, if gel stiffness is a known parameter. Such a model, may be potentially applicable not only to agarose gel substrates but to other 3D substrates as well.

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## 2. Materials and methods

### 2.1. Hydrogel fabrication

Agarose is a polysaccharide with alternating copolymers of 1,4-linked 3,6-anhydro- $\alpha$ -L-galactose and 1,3-linked  $\beta$ -D-galactose. Agarose is soluble in water at temperatures above 65°C and depending upon the degree of hydroxyethyl substitutions on its side chains, it gels in a range of 17–40°C. Once agarose gels, it is stable and does not swell at constant temperature or re-liquefy until heated to 65°C. SeaPrep® agarose is commercially available and forms an optically clear gel at 17°C. Neither peripheral nor central neurons have any known receptors that interact with agarose side chains. Agarose gels were fabricated in the range of 0.75–2.00% (wt/vol, increments of 0.25%), by dissolving powdered agarose in sterile saline.

### 2.2. Rheometry

In order to determine the mechanical stiffness of the gel, dynamic mechanical analysis using an RFS 8500 fluid rheometer (Rheometrics) was performed. Briefly, an oscillating parallel plate configuration was used to apply a shear strain to Seaprep® agarose gels ranging in concentration from 0.75 to 2.00% and the gel stress response was measured. Frequency range for the applied strain was 0.01–100 rad/s, and the obtained complex modulus ( $G^*$ ) measurements at frequencies between 0.1 and 10 rad/s (a stable plateau in the response) were used to approximate the equilibrium modulus  $G$ . The resistive force encountered by the growth cone due to gel stiffness was calculated by multiplying the equilibrium modulus by the approximated area of the tip of the growth cone. Student's  $t$ -test was used to determine statistical significance between the force values at different gel concentrations.

### 2.3. DRG culture

Pre-determined concentrations of agarose solutions were passed through a 0.22  $\mu$ m syringe filter for sterilization. Explants of DRGs were mixed with the agarose solution and this mixture was placed into 24-well tissue culture plates. The wells were then cooled at 4°C for 15 min to allow for gelation and entrapment of neurons in 3D. Dulbecco's culture medium (DMEM) with 10% fetal bovine serum (FBS), 50 ng/ml nerve growth factor (NGF) and 1% penicillin–streptomycin was added on top of the gels, and the wells are placed in a standard tissue culture incubator at 37°C, 100% humidity and 5% CO<sub>2</sub>. The cells were nourished with fresh medium in intervals of 48 h, and the rate of neurite extension was determined as described next.

### 2.4. Rate of neurite extension

E9 chick DRGs were embedded in 3D agarose gels and cultured for 24 h. Images were collected using an imaging station consisting of a Nikon Eclipse TE300 inverted microscope with phase contrast and Hoffman modulation optics and fluorescence capabilities. A Javelin color CCD camera connects the microscope to a PC, running the imaging software Scion Image (Scion Corp.), via the Scion LG-3 frame grabber for data acquisition. As only one plane of focus can be photographed at a time, all of the neurites in the images captured are not in focus due to 3D process extension. The imaging station described above was used for collecting time lapse video frames of neurite growth to determine the rate of neurite extension. Neurite growth was recorded for 2 h at the rate of 1 frame/min, 24 h after culture initiation.

### 2.5. Confocal imaging

Dissociated DRGs were fixed with 4% paraformaldehyde for 60 min at room temperature. Fixed cells were permeabilized with 0.02% Triton-X100 (Sigma) at room temperature for 45 min (1% Seaprep®) to 1 h (2% Seaprep®). The cells were incubated in Texas Red Phalloidin (Molecular Probes) for 45 min and washed with PBS. The samples were mounted with Prolong Antifade (Molecular Probes) and allowed to dry overnight. Cells were observed under a confocal microscope (Zeiss LSM-inverted, Axiovert 100). Images were analyzed with ImageSpace (Molecular Dynamics). For confocal microscopy, 35 mm culture dishes were prepared by drilling holes in the bottom of the dish and gluing glass cover slips over these holes. Three-dimensional gels with DRGs were cultured in these custom-made dishes to be compatible with the stage for confocal microscopy.

## 3. Results

### 3.1. Calculation of gel stiffness ( $G^*$ )

From rheometry, the hydrogel stress response was calculated using complex dynamic modulus ( $G^*$ ) measurements obtained from a frequency sweep in the range 0.01–100 rad/s. A representative sample of data obtained for 0.75% Ag is shown in Fig. 1. The magnitude of  $G^*$  was obtained from the stable plateau phase at frequencies between 0.1 and 10 rad/s.

### 3.2. Influence of gel concentration on the complex modulus, $G^*$

Fig. 2 shows that the equilibrium modulus increases with increasing gel concentrations, as the matrix become progressively stiffer ( $R^2 = 0.969$ ). A significant difference

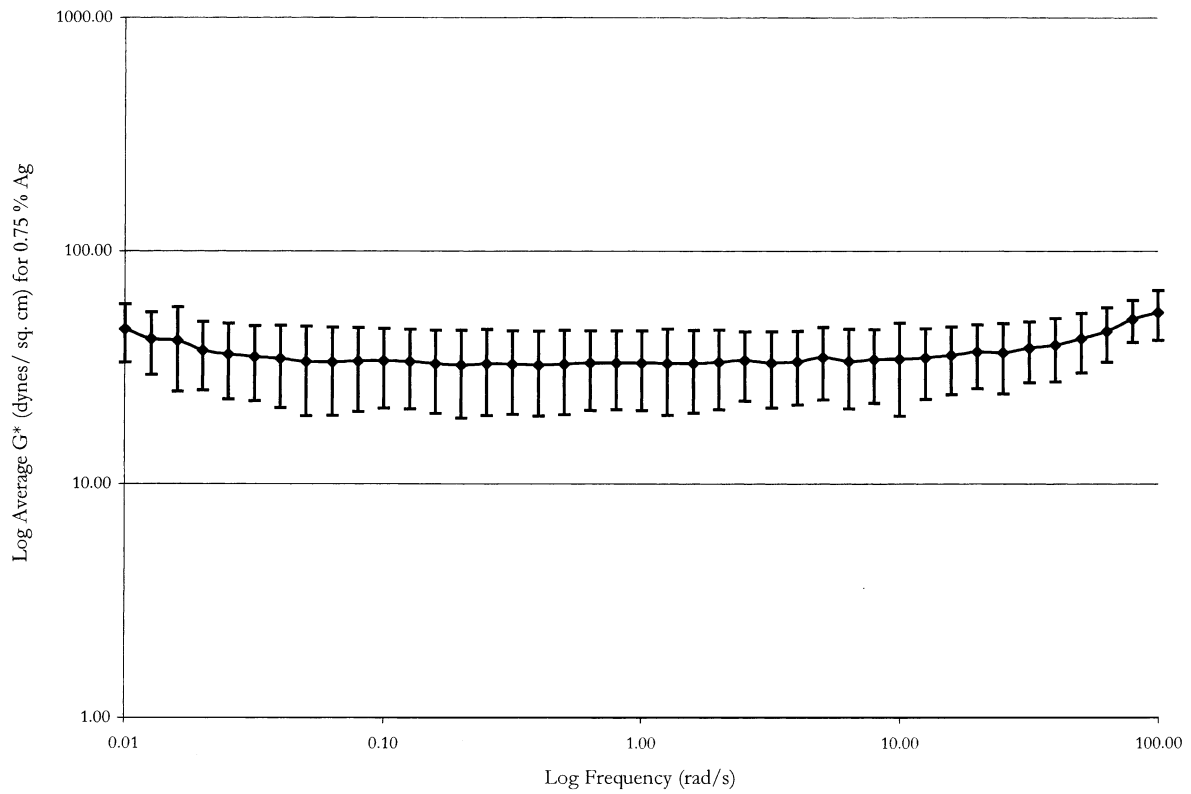


Fig. 1. Graphical plot of a “frequency sweep” of 0.75% agarose gel. Frequency range for the applied strain was 0.01–100 rad/s, but the equilibrium modulus was calculated from complex modulus ( $G^*$ ) measurements at frequencies between 0.1 and 10 rad/s (a stable plateau in the response). Similar plots were used to calculate  $G^*$  for all the concentrations of agarose gels used.

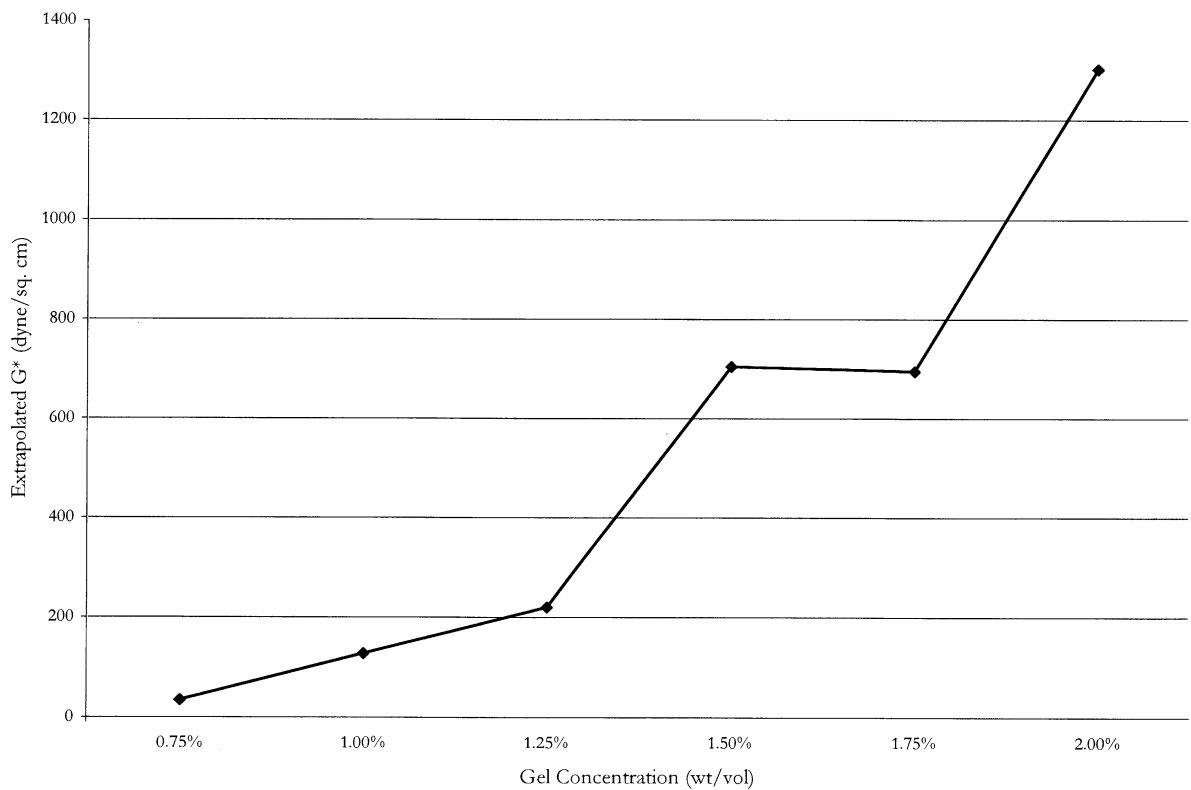


Fig. 2. Graphical plot of gel stiffness ( $G^*$ , dynes/sq cm) as a function of agarose gel concentration (wt/vol).

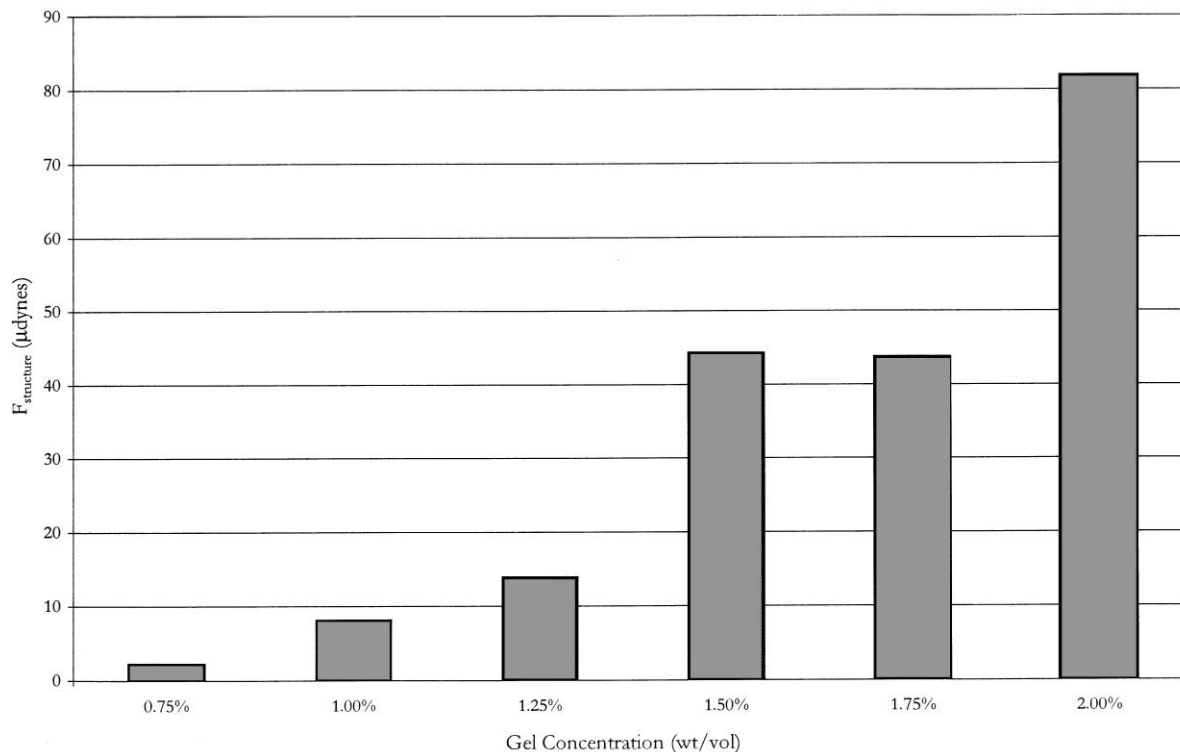


Fig. 3. Bar graph of force,  $F_{\text{structure}}$ , experienced by the extending growth cone in a range of agarose gel concentrations.

( $p < 0.05$ ) was found in the magnitude of modulus  $G^*$  between each pair of consecutive gel concentrations except for 1.5 and 1.75%. The magnitude of complex modulus  $G^*$  was used to compute the force exerted by the hydrogel network on the advancing neurite growth cone (Fig. 3). The force was obtained by the product of the magnitude of  $G^*$  and the contact area of the advancing growth cone.

### 3.3. Quantification of the rate of neurite extension

DRGs were cultured in agarose hydrogel of known concentrations as described above. After 24 h of culture, increase in neurite length was generally observed. Rate of neurite extension was quantified using time-lapse microscopy (Fig. 4). The rate of neurite extension decreases with an increase in gel concentration ( $R^2 = 0.994$ ). The difference in neurite growth rate between 1.00 and 2.00% agarose is statistically significant ( $p < 0.05$ ). Fig. 5 presents time-lapse images of DRG growth cone extension in 1.00% agarose gel.

### 3.4. Comparison of actual and predicted values of neurite extension

We postulate a general model for the prediction of rate of neurite growth that is of the form:

$$\text{Rate}_{\text{NE}} = dL/dt = S_g [T_n - (F_{\text{Structure}} \pm F_{\text{Interaction}})].$$

$\text{Rate}_{\text{NE}}$  is the rate of neurite extension which was experimentally determined using time-lapse video microscopy.  $S_g$ , the ‘growth sensitivity’ for chick neurons, is  $0.2\text{--}2.0 \mu\text{m}/(\text{h } \mu\text{dyn})$  [8,9].  $T_n$ , the typical tension the neuron is capable of developing intrinsically, is due to the microtubule assembly associated with normal axonal elongation and is typically between 100 and 150  $\mu\text{dyn}$  for primary sensory neurons [10].  $F_{\text{Structure}}$  is the resistive physical force due to gel stiffness and is quantifiable by measurements of gel elasticity, as shown below.  $F_{\text{Interaction}}$  is the force of attraction or repulsion between the neuron and the gel substrate. This force can represent or be modulated by the presence of charge on the agarose backbone. We used Matlab® (Mathworks) to determine the optimal values for  $S_g$  and  $T_n$  that best fit our experimental data. The range of  $S_g$  and  $T_n$  values tested were within the range previously determined experimentally [10]. We assume that  $F_{\text{interaction}} \sim 0$  (in uncharged gels),  $S_g = 0.2$  and  $T_n = 100$  to obtain the best possible fit. Implementing our model for neurite extension, the predicted values of neurite extension were determined (Fig. 7, Table 1). There is a significant correlation between the actual and predicted values of neurite extension ( $R^2 = 0.928$ ).

### 3.5. Calculation of “ $F_{\text{structure}}$ ”

Surface rendering of stacks of images obtained by confocal microscopy, allowed us to create a 3D image of

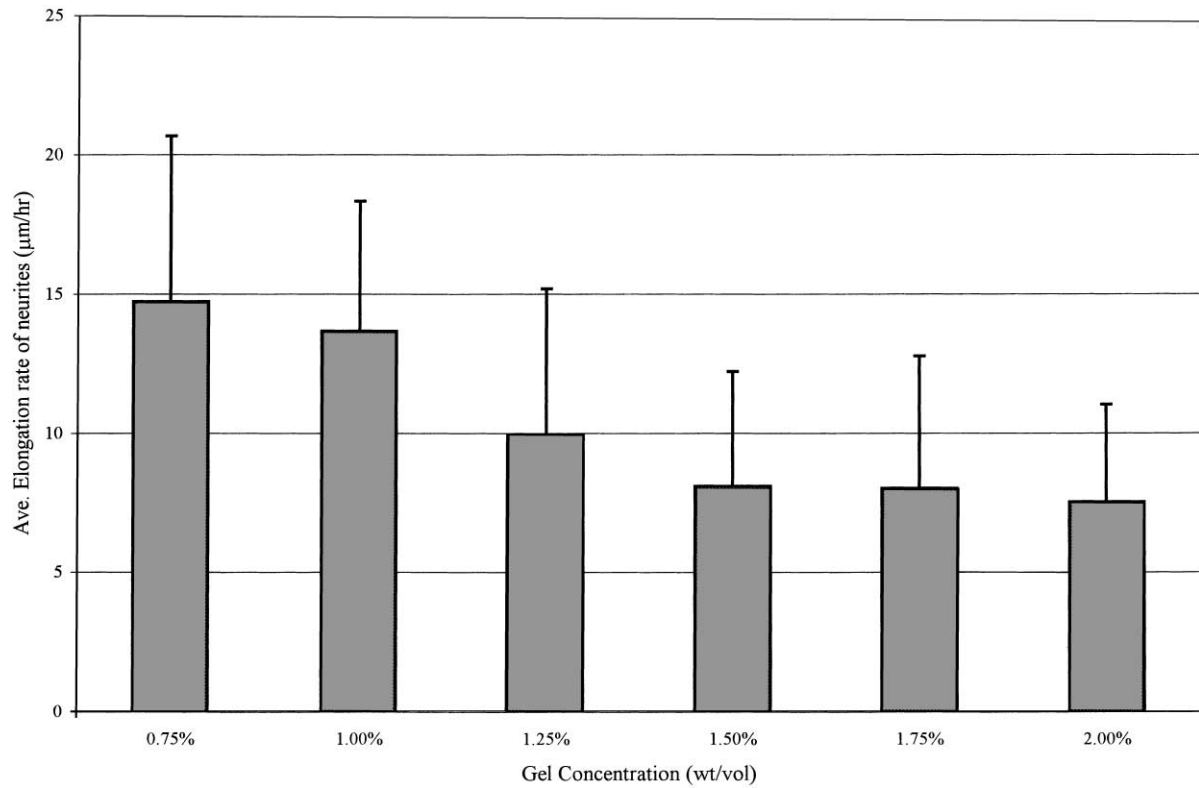


Fig. 4. Graphical plot of the rate of E9 chick DRG neurite extension as calculated in 0.75–2.00% agarose gel concentrations.

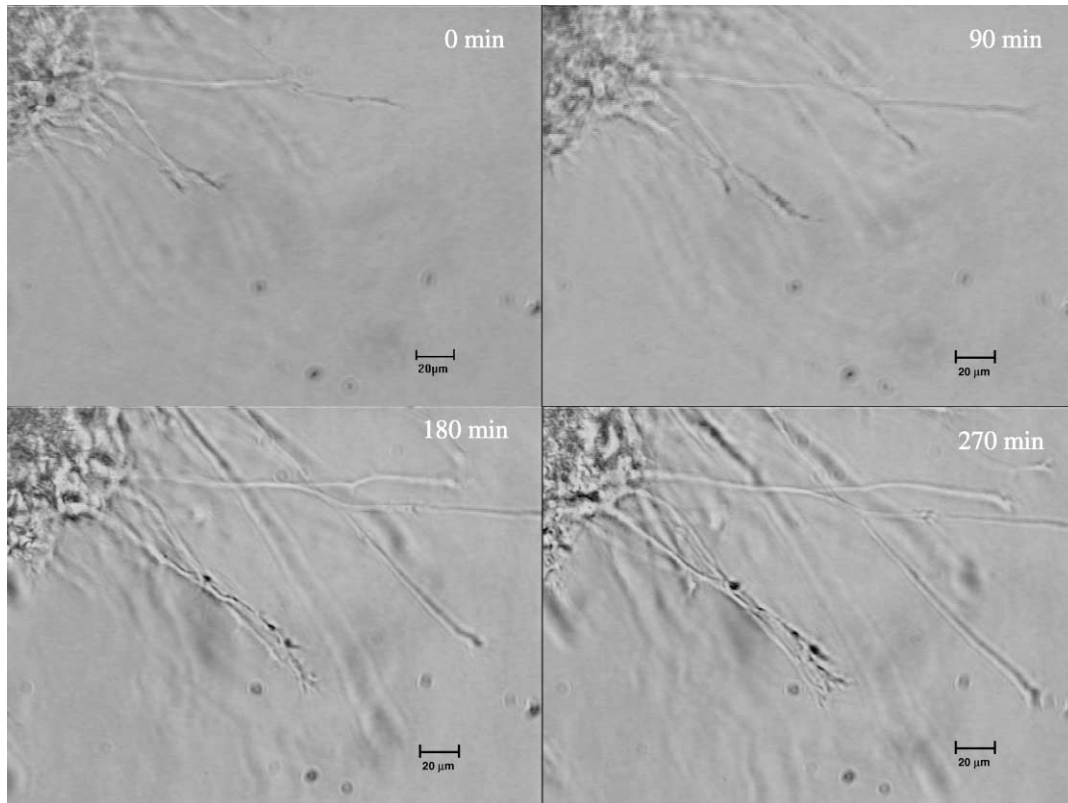


Fig. 5. Phase-contrast photomicrographs captured every 90 min of E9 chick DRG neurites extending growth cones in 1.00% agarose hydrogel. Magnification  $200\times$ .

Table 1  
Experimental and predicted rate of neurite growth in various gel concentrations

Agarose conc. (wt/vol)	0.75%	1.00%	1.25%	1.50%	1.75%	2.00%
Actual Rate <sub>NE</sub> (μm/h)	14.74	13.25	10.49	8.15	7.14	5.69
Predicted Rate <sub>NE</sub> (μm/h)	19.57	18.39	17.24	11.15	11.27	3.65

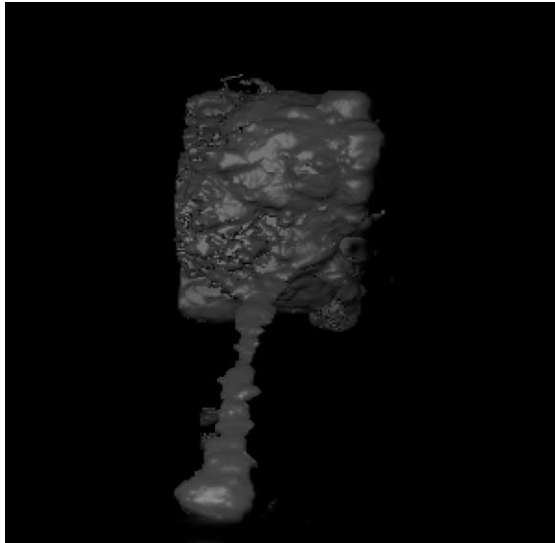


Fig. 6. Confocal image used to generate a surface rendered 3D image of a growth cone in 1.00% agarose gel.

the growth cone (Fig. 6). It is apparent that the growth cone assumes a bulbous morphology in a hydrogel environment. Based on this observation, we assumed that the shape of the growth cone can be approximated to that of a uniform sphere. Using light and video microscopy the radius of the DRG growth cone was determined to be  $\sim 1 \mu\text{m}$  in agarose hydrogel. Given that only the distal portion of the growth cone counters the primary mechanical barrier presented by the hydrogel during neurite extension, this hydrogel-interfacing surface of the growth cone was modeled after a hemisphere. It was assumed that the force exerted on the growth cone by the hydrogel network is countered by an area of the growth cone that is equivalent to the surface area of a hemisphere with radius  $1 \mu\text{m}$ .  $F_{\text{structure}}$  was determined by multiplying the magnitude of the complex modulus ( $G^*$ , dynes/sq cm) with the hemispherical area of a growth cone of radius  $1 \mu\text{m}$ . An increase in  $F_{\text{structure}}$  was correlated to an increase in gel concentration and a decrease in the rate of neurite extension (Figs. 3 and 7).

#### 4. Discussion

Reconstruction of soft tissues such as nerve is sensitive to the mechanical properties of the substrate [6,11]. Therefore, to maximize axonal regeneration for neural tissue engineering, it is important to construct scaffolds that have favorable mechanical properties. A 3D scaffold used for neurite regeneration can be optimized with respect to a variety of parameters. We have shown that the pore radius of agarose hydrogel increases with increased

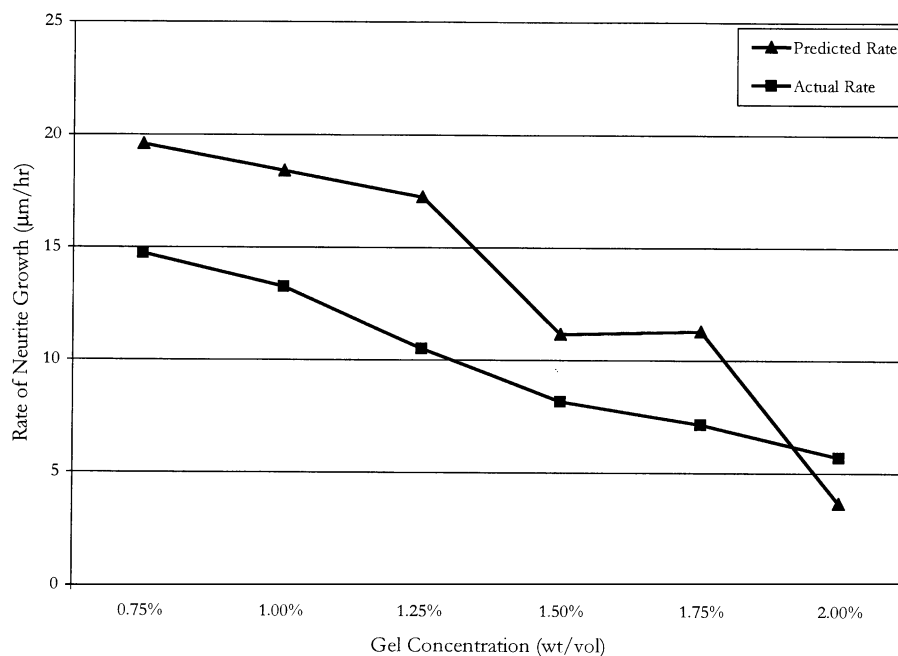


Fig. 7. Comparison of experimental and predicted rates of neurite extension to test the efficacy of the mathematical model ( $R^2 = 0.928$ ).

gel concentration and is inversely proportional to the length of DRG neurites [6]. In the present study, we explored the role of mechanical stiffness as a potential parameter than can be perturbed to enhance neurite extension in agarose hydrogel. The stiffness parameter of the hydrogel network was also employed in a mathematical model to predict the rate of neurite extension. An agarose hydrogel matrix thus optimized and analyzed with respect to its stiffness and ability to allow maximal neurite extension, would be an ideal candidate for use as a mechanically controlled 3D scaffold in neural tissue engineering.

The stiffness of the hydrogel as represented by the magnitude of complex modulus ( $G^*$ ) was chosen to quantitatively characterize the proclivity of the 3D matrix to permit neurite elongation. Using gels of agarose concentrations that favor neurite growth, we computed a range of corresponding  $G^*$ . The magnitude of  $G^*$  was in turn used to determine the force ( $F_{\text{structure}}$ ) exerted by the hydrogel upon the advancing growth cone. This calculation yields a range of  $F_{\text{structure}}$  values within which a measurable degree of neurite extension is observed. In addition, there is a strong inverse relationship between the force,  $F_{\text{structure}}$ , exerted by the hydrogel and the rate of neurite extension. Evidently, mechanical stiffness of the hydrogel is a good indicator of the ability of the agarose hydrogel to promote neurite extension.

Mechanical stiffness, as an indicator of neurite growth was further used in the development of a mathematical model based on Heidemann and Buxbaum's cyto-mechanical study of neurite growth [10]. Their studies suggest that growth cones of chick sensory neurons pull on their neurites and that neurite extension can be initiated and maintained by externally applied tension. Above a certain threshold tension ( $0 > 200 \mu\text{dyn}$  for chick sensory neurons), neurite elongation is observed [10]. In our mathematical model of neurite extension we interpret this externally applied tension as equivalent to the intrinsic tension ( $T_n$ ) generated by actin polymerization to overcome the mechanical barrier presented by the hydrogel. The value of  $T_n$  is believed to be between 100 and  $150 \mu\text{dyn}$  [10]. Tension threshold values for chick sensory neurons grown on different substrates were virtually identical, therefore, we can assume that  $T_n$  values for various concentration of gel would be nearly identical. Surface rendering of confocal microscopy images suggests a marked difference between morphologies of neuronal growth cones cultured in 3D hydrogels and 2D culture dishes. In 3D, the growth cone assumes a more globular form instead of the familiar lamellar morphology observed in 2D. Based on this observation, we assumed that the growth cone's morphology can be best represented in a mathematical model, by a sphere. The radius of this "sphere" was determined experimentally by measuring radii of growth cones cul-

tured in hydrogel. The growth cone was assumed to be similar to a uniform sphere of radius  $1 \mu\text{m}$ .

A mathematical model based on the above assumptions was implemented to predict the rate of neurite extension in agarose hydrogels of known concentrations. As shown in Table 1, our mathematical model ( $\text{Rate}_{\text{NE}} = dL/dt = S_g [T_n - (F_{\text{structure}} \pm F_{\text{interaction}})]$ ) is able to predict the rate of neurite extension for a given mechanical stiffness. These data have shown stiffness as a good predictor of the rate of neurite growth in 3D agarose hydrogels. Evidently, Heidemann and Buxbaum's cyto-mechanical theory of neurite extension can be employed to predict the rate of neurite extension if mechanical stiffness is known. It is not clear whether this model can be successfully applied to other 3D scaffolds used in neural tissue engineering. We are currently evaluating this model for its ability to predict neurite growth rates in collagen gels. Moreover, an important aspect of this study is the future application of the mathematical model to assess the mechanical properties of laminin and chondroitin sulfate coupled agarose gels [11], where the  $F_{\text{interaction}}$  parameter would be non-zero.

The differences between actual and predicted rates of neurite extension can be attributed to the various assumptions made in this model. The exact value of  $T_n$  for our system could be different and could thus affect the outcome of the mathematical model. Experiments to determine the precise value of  $T_n$  for our experimental model are in progress. Our model does not account for filopodial extension in its representation of the growth cone as a sphere. However, even in the presence of these assumptions, we believe that the ability to predict the rate of neurite extension in a range of agarose gel concentrations, on the basis of their mechanical stiffness is a very useful and important finding.

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