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Title

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<https://escholarship.org/uc/item/0hc336sx>

Journal

Biomaterials, 25(27)

ISSN

0142-9612

Authors

Stokols, S
Tuszynski, M H

Publication Date

2004-12-01

Peer reviewed



The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury

Shula Stokols^a, Mark H. Tuszynski^{b,c,*}

^a Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093, USA

^b Department of Neuroscience, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA

^c Veterans Affairs Medical Center, San Diego, CA 92161, USA

Received 18 November 2003; accepted 20 January 2004

Abstract

Strategies to promote axonal extension through a site of injury, including the provision of nervous system growth factors and supportive substrates, produce growth of axons, that is highly random and does not extend past the lesion site and into the host tissue (Brain Res. Bull 57(6) (2002) 833). Physically guiding the linear growth of axons across a site of injury, in addition to providing neurotrophic and/or cellular support, would help to retain the native organization of regenerating axons across the lesion site and into distal host tissue, and would potentially increase the probability of achieving functional recovery. In the present study, a novel procedure was developed for using freeze-dry processing to create nerve guidance scaffolds made from agarose, with uniaxial linear pores. The hydrated scaffolds were soft and flexible, contained linear guidance pores extending through their full length, were stable under physiological conditions without chemical crosslinking, and could be readily loaded with diffusible growth stimulating proteins.

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Keywords: Nerve guide; Regeneration; Scaffold; Polymer; Porous; Agarose

1. Introduction

Spinal cord injury affects 11,000 people per year in the US [1] and usually results in devastating and permanent loss of function. However, despite the inability of the CNS to naturally regenerate following injury, current experimental therapies have demonstrated that robust regeneration of axons can be achieved. Strategies to promote axonal extension through a site of injury include both the provision of nervous system growth factors and implantation of substrates to support axon extension, such as cellular grafts. In general, however, the growth of axons is highly random and does not extend past the lesion site and into host tissue [2]. Physically guiding the linear growth of axons across a site of injury, in addition to providing neurotrophic and/or cellular support, would help to retain the native

organization of regenerating axons across the lesion site and into distal host tissue, and would potentially increase the probability of achieving functional recovery.

The use of biopolymers can be a practical tool to provide neurotrophic and/or cellular support while simultaneously guiding axonal regeneration. Indeed, numerous natural and synthetic polymers, including poly-(α -hydroxyacids), collagen, fibronectin, and hyaluronic acid, have been used as scaffolds or within scaffolds for peripheral and central nerve regeneration, and have been reviewed in detail [3,4]. Many of the fabrication technologies for these polymers are based on particulate-leaching techniques, heat compression, and extrusion [5,6]. However, the harsh operating conditions of these processes can limit the incorporation of bioactive proteins and cells, and residual amounts of the chemical solvents required may cause toxicity in vivo.

Freeze/dry processing is an alternative method for producing porous scaffolds that does not require additional chemicals, relying instead on the water already present in hydrogels to form ice crystals that can be sublimated from the polymer, creating a

*Corresponding author. Department of Neuroscience, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA. Tel.: +858-534-8857; fax: +858-534-5220.

E-mail address: mtuszyns@ucsd.edu (M.H. Tuszynski).

particular micro-architecture. Because the direction of growth and size of the ice crystals are a function of the temperature gradient, linear, radial, and/or random pore directions and sizes can be produced with this methodology [7]. In the present study, a novel procedure was developed for using freeze-dry processing to create nerve guidance scaffolds made from agarose, with uniaxial linear pores. Previous *in vivo* studies from this lab have identified agarose, among several candidate biopolymers, as an optimal material to use in a nerve regeneration scaffold based on its biocompatibility, inertness, and stability in the spinal cord. The agarose scaffolds fabricated in this study, which could later be filled with extracellular matrix molecules and growth factors, were characterized for microstructure, water absorbability, *in vitro* degradation, biocompatibility, and growth factor loading.

2. Materials and methods

2.1. Scaffold preparation

Freeze-dry processing was used to create agarose scaffolds with linear pores. Agarose (Invitrogen, Carlsbad, CA) was dissolved at 30 mg/ml in distilled water, heated to 100°C, and injected into glass tubes of 8 mm diameter and 7.5 cm length (1 ml/tube). The tubes were centrifuged to remove air bubbles, and the agarose was allowed to cool to room temperature. The glass tubes containing agarose were placed in an insulating Styrofoam container, such that only the bottom surface of the glass tube was exposed. The glass tube in Styrofoam was then placed onto the surface of a 15 × 15 × 3 cm³ block of dry ice which in turn rested in a 3 cm-deep pool of liquid nitrogen, which created a uniaxial thermal gradient (Fig. 1). Liquid nitrogen vapor was removed by vacuum (500 mm Hg). The samples were allowed to freeze for 45 min, at which point linear ice crystals could be seen extending through the polymer, and were

lyophilized overnight. Scaffolds were removed from the tubes, and blocked with stainless-steel microtome blades into cylinders measuring 8 mm in diameter × 2 mm in length for further characterization.

2.2. Microstructure analysis

Scaffolds were sectioned in longitudinal or transverse planes and visualized by scanning electron microscopy (SEM) or light microscopy (LM). For SEM, sectioned scaffolds were attached to sample stubs, sputter-coated with gold/palladium, and visualized with a FEI Quanta 600 ESEM, using an accelerating voltage of 20 kV. For LM, both dry and hydrated scaffolds measuring 8 mm in diameter × 2 mm in length were placed on slides and visualized using phase contrast optics. Dry and hydrated pore dimensions were directly measured from light micrographs. The shortest and longest pore dimensions were measured in a sample of 60 pores from three different scaffolds (20 pores measured per scaffold). Effective diameter of each pore was expressed as the mean of its shortest and longest dimensions. Results are expressed as mean pore size ± standard deviation. To assess whether pores extended through the full length of the scaffolds, two methods were used. First, individual scaffolds of 1-cm length were positioned vertically on filter paper, and 15 ul of water was placed onto the top surface using a 20 ul pipette. The examiner observed the scaffold on filter paper and scored whether fluid emerged from the channel only from the distal end of the cylinder, from the walls of the channel, or both. This fluid egress measurement was performed 5 times using 5 identically fabricated scaffolds. Second, light micrographs of the proximal and distal surfaces of scaffolds 2 and 4 mm in length (3 scaffolds for each length) were compared to determine whether individual pore configurations matched on the proximal and distal surfaces of the scaffold (see Figs. 2D-G).

2.3. Water absorption

Mass water absorption was measured daily over 7 consecutive days. The dry weight of three scaffolds of 8 mm diameter and 2 mm length were measured, then scaffolds were immersed in 1 ml of phosphate buffered saline (PBS) (pH 7.4). To determine wet weight, scaffolds were removed from PBS, lightly blotted onto filter paper until adsorbed water was removed (achieved when water was no longer visibly transferred to the filter paper), and immediately weighed. The percent mass increase due to water absorption was calculated from the expression

$$M_i = (M_w - M_d) / M_d \cdot 100$$

where M_i is the percent mass increase, M_w the wet mass and M_d the dry mass.

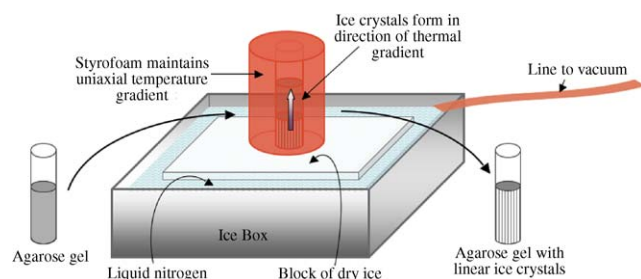


Fig. 1. A uniaxial temperature gradient was created by exposing the bottom surface of glass tubes containing polymerized agarose to a block of dry ice cooled in liquid nitrogen. The glass tubes were insulated in Styrofoam, and liquid nitrogen vapor was removed by vacuum (20 in Hg) to ensure a uniaxial temperature gradient. Arrow indicates direction of temperature gradient.

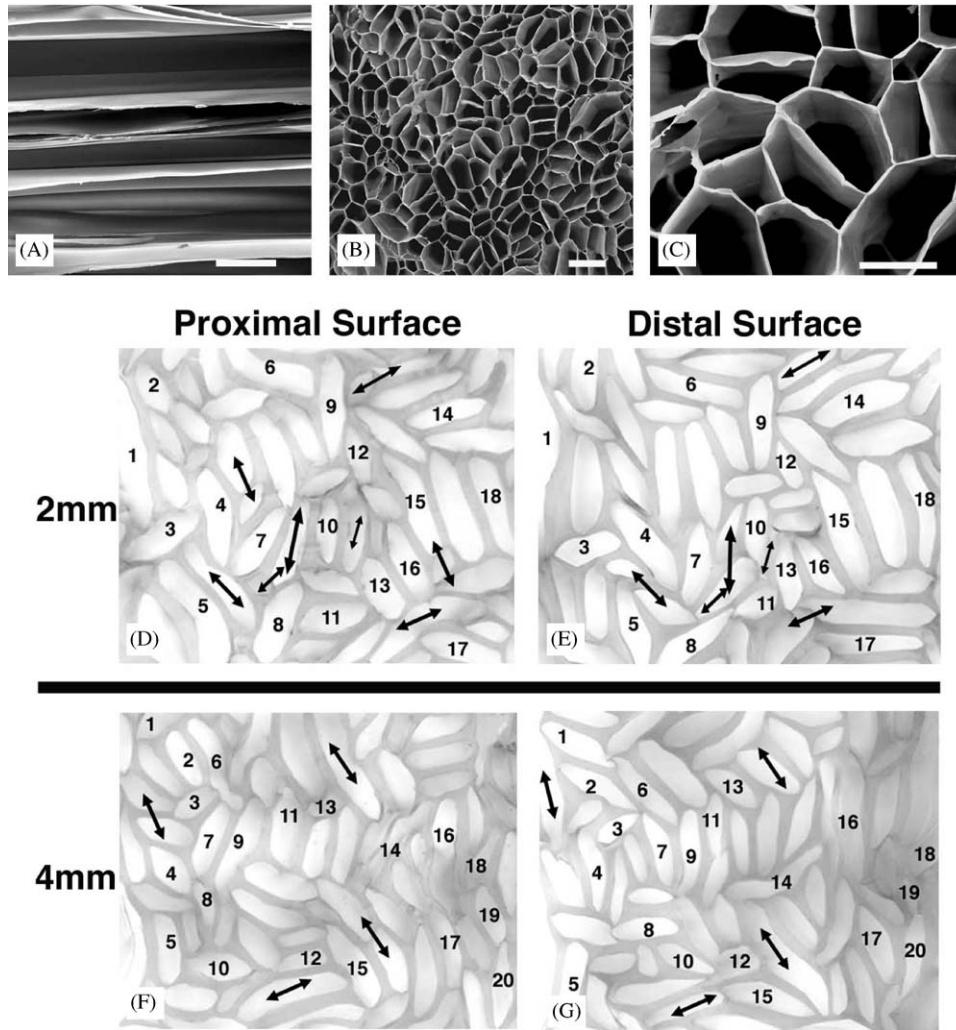


Fig. 2. (A–C) SEM images of scaffold sectioned longitudinally (A; scale bar 200 μm) or cross-sectioned (B; scale bar 200 μm and C; scale bar 100 μm). A highly linear configuration is evident. (D–G): Light microscopic images of proximal and distal surfaces of scaffolds of 2 mm (D, E) and 4 mm (F, G) length. Numbers indicate identical pores at top and bottom surfaces of scaffold; arrows indicate pores that have joined or split.

Results are expressed as the percent mass increase of the scaffold \pm SEM. In addition, change in wall thickness due to water absorption was determined. Dry and hydrated wall dimensions were directly measured from light micrographs. For each condition, a total of 18 walls from three different scaffolds (6 walls per scaffold) were measured. Results are expressed as the mean wall thickness \pm standard deviation.

2.4. Hydrolysis/degradation

Mass degradation of hydrated scaffolds was measured over a 1-month period. Fifteen dry scaffolds of 8 mm diameter and 2 mm length were weighed and then immersed in 1 ml PBS (pH = 7.4). Three scaffolds were removed from the water bath every 5 days, lightly blotted on filter paper until all adsorbed water was removed, placed in a 60°C oven for 24 h to remove all absorbed water, and then reweighed. The percent of

original mass remaining was calculated from the expression

$$R = (M_f - M_o) / M_o \cdot 100,$$

where R is the percent mass remaining, M_o the original mass and M_f the final mass.

Results are expressed as the percent of original mass \pm SEM.

2.5. Growth factor loading, release, and bioactivity

Nerve growth factor (NGF) was used as a model growth factor to assess scaffold growth factor loading, release, and bioactivity properties. PC12 cell cultures were used to assay the bioactivity of NGF released from scaffolds. Recombinant NGF protein was included in the scaffolds in one of two ways. In the first method, the pores of the scaffold were injected with polymers of extracellular matrix molecules that have previously been

identified as potential substrates for axonal regeneration: collagen (3 mg/ml) or hyaluronic acid (40 mg/ml). Prior to injection into the scaffolds, 3 ng NGF (100 ng/ml Genetech) was mixed into 30 μ l of collagen or HA solution. In the second method, NGF (300 ng/ml) was added to the agarose prior to polymerization and freeze-dry processing, allowing NGF incorporation into the scaffold material itself (30 ng/scaffold). Both types of NGF-containing scaffolds were placed in a sterile incubator at 37°C for 1, 2, 3 or 4 weeks. PC12 cells were seeded onto collagen coated 12-well plates and allowed to adhere for 2 h. One ml of RPMI media + 10% horse serum + 5% fetal bovine serum was added to each well. For each time point and each type of NGF-containing scaffold, a scaffold was placed inside a well containing PC12 cells. For a positive control, 3 ng of recombinant NGF protein was added directly to the medium without a scaffold. For a negative control, an agarose scaffold lacking NGF was added to the well. For an additional negative control, neither scaffold nor growth factor were added to the well. After 24 h, neurite extension was evaluated by quantifying the number of cells extending neurites greater than one cell diameter. For each condition, 3 wells were counted, consisting of at least 100 cells per well.

2.6. Biocompatibility

In vitro biocompatibility was tested by co-incubation of scaffolds with 293-T cells. Scaffolds were sterilized by sequential immersion in 100% ethanol, 70% ethanol, and 50% ethanol for 1 h each, and were then equilibrated and stored in sterile water. Prior to use, they were lightly blotted on sterile filter paper to remove all adsorbed water. 293-T cells were plated at ~50% confluency on 48 well culture plates. One scaffold was placed inside each well containing 2 ml of DMEM media + 5% Fetal Bovine Serum. The scaffold was positioned such that it was fully submerged in media, but not physically contacting cells. Media was changed every other day, and rate of cell proliferation was qualitatively compared to control wells. Evidence of cytotoxicity (rounding of cells and lifting from the well surface), if present, was recorded in 8 separate wells.

3. Results

The linear uniaxial freezing gradient produced final scaffolds with linear pores (Fig. 2A) in a honeycomb arrangement (Figs. 2B and C). The pores had a mean cross-sectional diameter of $119 \pm 26 \mu\text{m}$, range = 71–187 μm .

Pores extended linearly through the full extent of scaffolds, evidenced by two separate measures. First, egress of fluid that was injected into one end of the

channel was examined at the opposite end. In each case ($n = 5$ scaffolds examined), fluid was discharged only from the opposite end of the channel and did not emerge from the side walls. In a second assay of channel continuity, photomicrographs of proximal and distal ends of 2 and 4 mm length scaffolds were compared for persistence of each channel at both ends of the scaffold. While as to be expected, corresponding proximal and distal channel openings were easier to detect with the shorter length scaffolds, 100% persistence of individual channel patency was found in both 2 and 4 mm-long scaffolds (Figs. 2D–G).

Immersion of scaffolds in PBS resulted in an increase in mass of $423 \pm 17\%$ within 24 h, and mass remained relatively constant during the remainder of the 1-week period tested (Fig. 3A). Wall width increased from 2.1 ± 0.1 to $9.0 \pm 0.7 \mu\text{m}$, yet the mean pore diameter was essentially unchanged: 125 ± 25 and $119 \pm 26 \mu\text{m}$ for the hydrated and dry scaffolds, respectively (Figs. 3C–F). Additionally, scaffolds did not exhibit signs of mass degradation due to hydrolysis over the 1-month period tested (Fig. 3B).

The biocompatibility of agarose, previously reported in both in vitro and in vivo paradigms [8–10], was confirmed using 293-T cell cultures into which sterilized scaffolds were placed for 48 h. The 293-T cells showed no evidence of cytotoxicity (loss of adherence, nuclear condensation, cell soma contraction) and cells proliferated normally compared to cells in control wells, expanding from approximately 50–100% confluency within 72 h.

The ability of the scaffolds to function as depots for growth factors was evaluated either by (1) filling the pores of the scaffold with NGF incorporated in collagen or hyaluronic acid matrices, or (2) by incorporating NGF in agarose before the latter was polymerized and freeze-dried. Both types of NGF-containing scaffolds were incubated at 37°C for up to 4 weeks before being added to PC12 cell cultures. In the presence of NGF-containing scaffolds, the proportion of PC12 cells extending neurites ($43 \pm 8.1\%$ $p < 0.001$) was indistinguishable from the proportion of cells extending neurites when NGF protein was added directly to the medium ($47 \pm 6.8\%$; Figs. 4 and 5). In contrast, few PC12 cells extended neurites in the absence of NGF ($5.0 \pm 3.1\%$).

4. Discussion

This report describes the fabrication and characterization of freeze-dried agarose scaffolds with uniaxial linear pores extending through their full length. Theoretically, scaffolds created for guiding axonal regeneration should have pores small enough to physically align and restrict the direction of growing axons, yet large enough to allow for vascularization and the infiltration

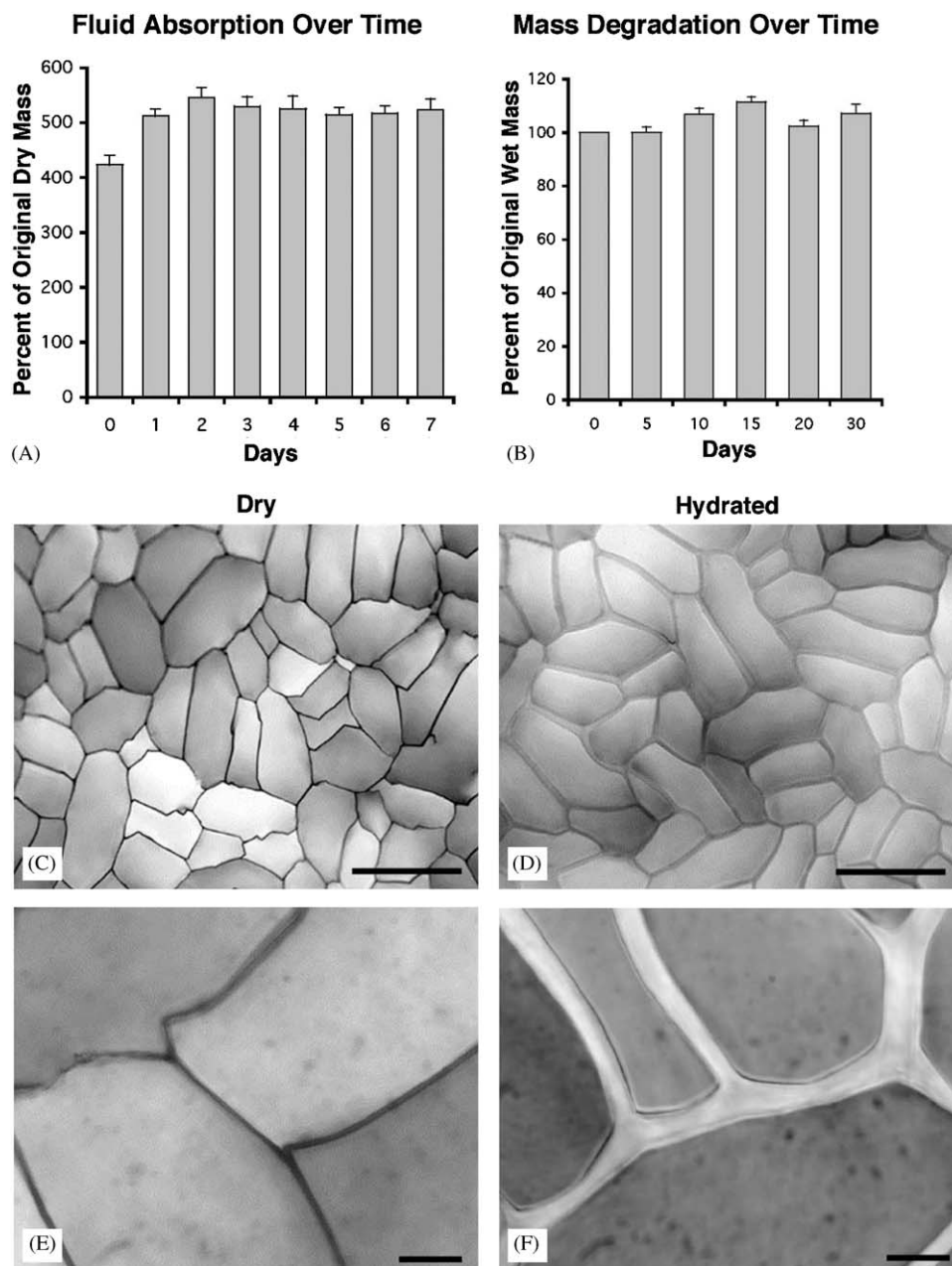


Fig. 3. (A) Water absorption determined daily over 7 days. There was a mean mass increase of $423 \pm 17\%$ during the first 24 h, which remained constant thereafter. (B) Mass degradation in PBS at 37°C . Scaffolds do not exhibit mass degradation due to hydrolysis over the 1-month period tested. (C–F): Light microscopic images of scaffold cross sections in dry and hydrated states. The scaffold ultrastructure and mean effective pore diameter are unchanged in the two conditions. Scale bar C, D 200 μm ; E, F 20 μm .

of cells which might support regeneration. To meet these requirements, we targeted production of a hypothetically ideal pore size between 100 and 200 μm . Although freeze-dry processing does not allow for precise control of pore size and shape, variations of the magnitude and direction of the temperature gradient can produce a range of different pore sizes and orientations. Dry ice alone did not establish a thermal gradient large enough to generate pores consistently smaller than 200 μm . However, when dry ice was combined with liquid nitrogen, a steeper thermal gradient was established,

resulting in pores of mean cross-sectional diameter $119 \pm 26 \mu\text{m}$, range 71–187 μm . In addition, the honeycomb arrangement of polygon shaped pores, which naturally resulted from the freeze-dry processing in the current preparation, achieves a maximal ratio of pore volume to wall volume (area available for regeneration).

Previously, synthetic poly(α -hydroxyacid) polymers, which have been used to create outer shells and internal structures of nerve guidance channels, have been shown to become brittle as they degrade, and collapse or break down into large polymer pieces which can compress

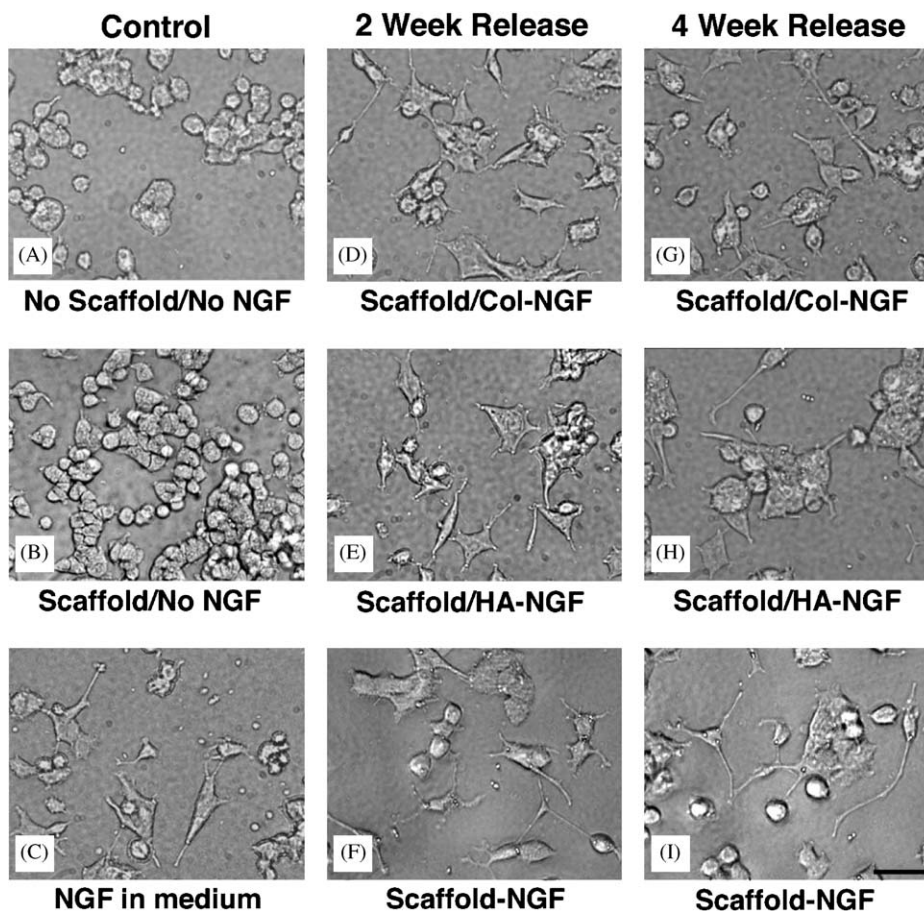


Fig. 4. Bioactivity of growth factor incorporation into channels. Scaffolds were loaded with NGF either by filling pores of the scaffold with polymer matrices containing 3 ng NGF, or by incorporating NGF into agarose before polymerization and freeze drying (30 ng/scaffold). Both types of NGF-containing scaffolds were incubated at 37°C for up to 4 weeks and were then added to PC12 cell cultures. (A) No NGF; (B) scaffold without NGF; (C) recombinant NGF protein (3 ng/ml) added directly to well; (D,G) scaffold with hyaluronic acid + NGF; (E,H) scaffold with collagen + NGF; (F,I) scaffold with NGF incorporated in agarose. Scaffolds released biologically active NGF over the time periods tested. Scale bar = 44 μ m.

regenerating tissue [11]. In contrast, natural polymer hydrogels, such as agarose used in this study, are soft and flexible polymers with mechanical properties more hypothetically advantageous for use in the spinal cord. Various formulations of PMMA hydrogels [12,13], collagen [14–17], and alginate [18,19], a polysaccharide similar to agarose, have been fabricated into porous scaffolds and gels. While some of these scaffolds exhibit biocompatibility and an ability to support axonal regeneration in both peripheral and CNS injuries, few of these scaffolds have been fabricated with individual linear pores that extend through their full length, a feature of practical necessity to guide nerve regeneration. In addition, collagen hydrogels are chemically crosslinked to avoid degradation due to hydrolysis, which may introduce toxic molecules into the site of injury.

Because regeneration of nerve tissue can require weeks to months, it is important that the physical structure of a guidance scaffold remain stable over that time period. The effects of *in vitro* water absorption and

degradation of the present scaffolds were evaluated over a 1-week and 1-month period, respectively. Upon immersion in PBS, the freeze-dry agarose scaffolds initially absorbed an average 423% their mass in water. However, apart from an increase in wall thickness from 2 ± 0.1 to $9 \pm 0.7 \mu$ m, their microstructure was unchanged. After the initial 24-hour period, there was no additional water absorption. Although many natural biopolymers degrade quickly in an aqueous environment, throughout the 1-month period tested the agarose scaffolds showed no signs of degradation due to hydrolysis.

Because agarose itself is a biologically inert material, it is important that scaffolds fabricated from this material have the capability to incorporate and release substances that may augment axon regeneration. Indeed, scaffolds released nerve growth factor (a model neurotrophic factor) with retention of biological activity for at least 4 weeks when it was polymerized within extracellular matrix molecules filling the channels of the scaffold and within the agarose walls.

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