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A pilot study of poly(*N*-isopropylacrylamide)-*g*-polyethylene glycol and poly(*N*-isopropylacrylamide)-*g*-methylcellulose branched copolymers as injectable scaffolds for local delivery of neurotrophins and cellular transplants into the injured spinal cord:

Laboratory investigation

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Abstract

Object—The authors investigated the feasibility of using injectable hydrogels, based on poly(*N*-isopropylacrylamide) (PNIPAAm), lightly crosslinked with polyethylene glycol (PEG) or methylcellulose (MC), to serve as injectable scaffolds for local delivery of neurotrophins and cellular transplants into the injured spinal cord. The primary aims of this work were to assess the biocompatibility of the scaffolds by evaluating graft cell survival and the host tissue immune response. The scaffolds were also evaluated for their ability to promote axonal growth through the action of released brain-derived neurotrophic factor (BDNF).

Methods—The in vivo performance of PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC was evaluated using a rodent model of spinal cord injury (SCI). The hydrogels were injected as viscous liquids into the injury site and formed space-filling hydrogels. The host immune response and biocompatibility of the scaffolds were evaluated at 2 weeks by histological and fluorescent immunohistochemical analysis. Commercially available matrices were used as a control and examined for comparison.

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Disclosure

Author contributions to the study and manuscript preparation include the following. Conception and design: all authors. Acquisition of data: Conova. Analysis and interpretation of data: Vernengo, Conova, Jin, Himes. Drafting the article: Conova. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Vernengo.

Results—Experiments showed that the scaffolds did not contribute to an injury-related inflammatory response. PNIPAAm-*g*-PEG was also shown to be an effective vehicle for delivery of cellular transplants and supported graft survival. Additionally, PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC are permissive to axonal growth and can serve as injectable scaffolds for local delivery of BDNF.

Conclusions—Based on the results, the authors suggest that these copolymers are feasible injectable scaffolds for cell grafting into the injured spinal cord and for delivery of therapeutic factors.

Keywords

hydrogel; biocompatibility; spinal cord injury; cell transplantation; growth factor; immune response

INJURIES to the CNS typically result in permanent functional loss due to the inability of the CNS to regenerate damaged axons.^{21,45,48} Current therapeutic strategies that have emerged for SCI treatment include delivery of neurotrophic factors, cellular transplants, and the use of synthetic scaffolds or matrices to promote neuronal regeneration and functional recovery.^{34,35} Cell-based strategies focus on replacing lost or damaged cells at the injury via cell transplantation of embryonic stem cells,²⁴ Schwann cells,³⁷ or olfactory ensheathing cells.²⁹ Neurotrophic factors have been shown to promote neural survival and axon regeneration. For example, neurotrophin-3 or BDNF delivery to the spinal cord promotes axon growth and plasticity.^{6,13,36,38,43,47,50} Encapsulation of these proteins in biodegradable sustained-release microspheres has also been studied for delivering and maintaining therapeutic levels of growth factors to the injury.^{1,2,39}

Scaffold-based approaches involve the use of a polymeric material to fill an injury gap. Many polymeric materials are composed of biodegradable polyesters, such as poly(lactic acid) and polycaprolactone.^{5,8,26,31,39} While these materials exhibit good biocompatibility, the preformed nature of these materials makes them difficult to implant into an injury site, and they are mechanically mismatched with spinal cord tissue. Crosslinked polyacrylamide gels of different densities have been studied as scaffolds, and it was found that cells grown on soft gels developed larger numbers of neuritic branches than did cells grown on stiffer polymers,⁹ suggesting that mechanical parameters may have an important effect on cell growth.

The long-term goal of this work is to develop a device that combines these cellular, mechanical, and biochemical strategies into one multifunctional platform. Specifically, this study investigates a hydrogel based on PNIPAAm lightly crosslinked with PEG or MC. Aqueous solutions of PNIPAAm have an LCST around 32°C, allowing them to be injected as a viscous liquid at room temperature and solidify in situ without the use of toxic monomers or cross-linkers.⁵¹ In our prior work, it was found that copolymerization of NIPAAm with hydrophilic macromers, such as methacrylated PEG, increases the elastic response and minimizes syneresis of the hydrophobic PNIPAAm chains above the LCST,⁵¹ therefore increasing gel porosity and the ability to accommodate cells. In fact, the mass and volume retention in vitro of PNIPAAm-*g*-PEG copolymers in DMEM with F-12 media with

serum, as well as PBS, at physiological temperature was shown to be close to 100%, making these scaffolds acceptable for tissue engineering implants.⁷ PNIPAAm-*g*-PEG was also shown to have compressive mechanical properties similar to the host tissue and can support the survival of seeded mesenchymal stem cells for at least 30 days in vitro.⁷ Additionally, codissolved BDNF is released in bioactive form from PNIPAAm-*g*-PEG hydrogels with a minimal burst, followed by a linear release for 4 weeks.⁷

Many recent studies on scaffolds in neural tissue engineering have focused on natural biopolymers such as proteins and polysaccharides.^{11,19,30,44} Such molecules have gained significant attention because of their intrinsic biocompatibility, given that they comprise the natural extracellular matrix, providing structure and support to cells, and also possess biodegradability. In vitro studies have shown that laminin-functionalized MC hydrogels⁴⁴ show good cell attachment and viability, and these are being studied as delivery vehicles to the injured CNS. Gupta et al.¹¹ demonstrated that the biocompatibility of MC blends with hyaluronic acid within the intrathecal space. Thus, in the present study, we chose to evaluate the in vivo performance of semisynthetic PNIPAAm-*g*-MC and completely synthetic PNIPAAm-*g*-PEG networks.

Our experiments showed that the PNIPAAm-*g*-PEG scaffold could be successfully injected into an injury site and gelled in situ to fill and remain in a spinal cord lesion, without contributing to an injury-related inflammatory response. PNIPAAm-*g*-PEG was also an effective vehicle for the delivery of cellular transplants and supported graft survival. Both PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC show good biocompatibility, are permissive to axonal growth, and are efficient injectable scaffolds for local delivery of BDNF in vivo. These preliminary results suggest that PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC are feasible for use as multifunctional injectable scaffolds for cell grafting into the injured spinal cord combined with controlled release of therapeutic factors.

Methods

PNIPAAm-g-PEG and PNIPAAm-g-MC Branched Copolymers Synthesis

The PNIPAAm-*g*-PEG copolymers were prepared as described previously.⁵¹ Briefly, NIPAAm monomer was polymerized in methanol in the presence of PEG (8000 g/mol) dimethacrylate at a molar ratio of NIPAAm monomer units to PEG blocks of 1000:1. This ratio was chosen because it produced highly elastic PNIPAAm-*g*-PEG hydrogels at physiological temperature, with suitable volume retention over time and minimum liquid viscosity in water below the LCST due to the limited crosslink density.

Methylcellulose (Methocel 65, Dow) was methacrylate-functionalized in an aqueous reaction with MA based on a previously described method⁴ using a molar ratio of MA:MC of 25:1. Next, an NIPAAm monomer was polymerized in the presence of MC methacrylate in water with ammonium persulfate as the initiator and tetramethylethylenediamine (TEMED, Fluka Co.) as the accelerator. Ammonium persulfate and TEMED were added in amounts corresponding to 8 wt% and 0.8 wt% of total monomer in solution, respectively.²³ NIPAAm and MC macromer were combined in the reaction mixture in a molar ratio of 6000:1. This molar ratio was chosen because it resulted in the minimum possible viscosity

of aqueous PNIPAAm-*g*-MC solutions at room temperature, while also limiting PNIPAAm syneresis and producing gels with a high elastic response.

Preparation of RSFs Infected With GFP and BDNF Cell Suspension

Primary RSFs were isolated as previously described.²² The cells were then infected in vitro with a lentiviral construct containing the GFP reporter gene and BDNF (RSFGFP BDNF). After infection, approximately 50% of the cells expressed GFP. The cells were cultured as described previously,²⁷ using DMEM (Gibco, Invitrogen) with 10% fetal bovine serum (HyClone Laboratories, Inc.) and 1% Antibiotic-Antimycotic (Anti-Anti) Solution (Cellgro, Mediatech, Inc.) containing 10,000 IU/ml penicillin and 10,000 µg/ml streptomycin. The cells were thawed 12 days prior to surgery, and 1.2×10^6 viable cells were plated (p3) on 3 sterile petri dishes. The cells were split and replated 1 week prior to surgery (p4) and again at 4 days prior to surgery (p5). The day of surgery, the cells were passaged (p6), and the total number of viable cells was 3.05×10^6 . The cells were then suspended in an appropriate amount of DMEM/10% fetal bovine serum media to achieve a concentration of 3.0×10^6 cells/µl. After completion of the surgery, cell viability was greater than 90%, as determined by a trypan blue assay.

Preparation of the Hydrogels, Gelfoam, and Vitrogen PureCol Solutions for In Vivo Implantation

After synthesis and purification, PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC copolymers were dissolved in DMEM medium (Gibco, Invitrogen) and steam-sterilized prior to implantation. The groups studied in vivo are summarized in Table 1. Group 1a received a PNIPAAm-*g*-PEG copolymer alone. Group 1b received Gelfoam sterilized by ultraviolet radiation and hydrated in sterile saline prior to implantation. For Group 2, an RSF-GFP BDNF cell suspension was added to the PNIPAAm-*g*-PEG polymer and mixed gently, to give a final concentration of 5.0×10^4 cells/µl. For Groups 3, 4 and 5, BDNF (PeproTech) was reconstituted and codissolved with PNIPAAm-*g*-PEG, PNIPAAm-*g*-MC, or Vitrogen PureCol, respectively. The final BDNF concentration in these samples was 0.05 µg/µl. The addition of BDNF diluted the PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC solutions to 10 wt% and 8 wt%, respectively. These concentrations were chosen because they were the lowest concentrations that produced solid gels above the LCST of PNIPAAm. All solutions were kept on ice prior to implantation.

Animal Surgery

In vivo studies were performed in adult female Sprague-Dawley rats, weighing approximately 225–250 g. All pre- and postoperative care was conducted in strict accordance with the guidelines established by the National Institutes of Health, the Drexel University College of Medicine Institutional Animal Care and Use Committee, and the Institute of Laboratory Animal Resources, US National Academy of Sciences. Starting 3 days before grafting and continuing throughout the postoperative survival period, animals in Group 2 received the immunosuppressant cyclosporin A (Sandoz Pharmaceuticals) via daily subcutaneous injections. Each animal received a cyclosporin A dose of 10 mg/kg of body weight. Cyclosporin A can also act as a neuroprotective agent after SCI, as it can depress

cellular and humoral immune responses by inhibiting T helper lymphocyte proliferation.^{17,18,32}

For Groups 1a and 1b, the in vivo performance of PNIPAAm-*g*-PEG and Gelfoam were evaluated in a partial hemisection at C4–5 levels. Animals were anesthetized with an intraperitoneal injection of an analgesic cocktail (acepromazine maleate [0.7 mg/kg, Fermenta Animal Health]), ketamine (95 mg/kg, Fort Dodge Animal Health), and xylazine (10 mg/kg, Bayer). The surgical lesion was generated at the C4–5 level,²⁵ as it creates a cavity that is a desirable model for a space-filling hydrogel design. Briefly, after cleaning the skin with Xenodine topical antiseptic (Bipore, Inc.), incisions were made through the muscle and skin, which were retracted. A laminectomy was performed to expose the right half of the C4–5 spinal cord. An aspiration lesion removed the white matter of the lateral funiculus, creating a cavity 2–3 mm in length. In Group 1a, each cavity was filled with 3 μ l of polymer solution, using a physical displacement pipette to minimize bubbles. For animals in Group 1b, hydrated Gelfoam sponge was implanted into the lesion. Following implantation, the dura, muscle, and skin were surgically closed.

In Groups 2–5, a model of dorsal root generation was used to analyze regeneration of dorsal roots into the matrices being tested.⁴⁹ Animals were anesthetized using 4% isoflurane in oxygen (1 L/minute), and anesthesia was maintained during surgery with 2.5% isoflurane in oxygen. The skin was cleaned and treated with Xenodine topical antiseptic. Incisions were made through the muscle and skin, which were then retracted. Laminectomy of the T-13 and L-1 vertebrae exposed the lumbar enlargement (L4–5 segments). The dura was cut along the mid-line, and the connected right dorsal roots were cut and reflected to expose the spinal cord. Gentle aspiration in combination with microscissors was used to create a right side partial hemisection cavity 2–3 mm in length. Sutures were placed in the dura on both sides of the lesion but not tightened. After achieving hemostasis in the spinal cord lesion, approximately 6 μ l of PNIPAAm copolymer solution or Vitrogen PureCol was injected directly into the cavity using a physical displacement pipette. The cut dorsal roots were juxtaposed to the graft, and the dura sutures tightened to close up the lesion and keep the solution within the lesion site. The muscle was then sutured and the skin was stapled closed.

Tissue Collection for Histological Analysis

The rats were killed at 1 week in Group 1 and at 2 weeks in Groups 2–5. The method of perfusion depended on the goal of the study. Animals in Group 1 were killed, flushed with 0.9% saline, then transcardially perfused using 4% PFA in 0.1 M phosphate buffer, pH 7.4 (Fisher Scientific). Since the aim of our initial study with Group 1 was to verify that the hydrogel remains in and fills the spinal cord lesion, the saline and PFA were maintained at 37°C to preserve the integrity of the thermosensitive polymer. However, in subsequent studies, we found that this perfusion method dislodged cellular grafts within the hydrogel. Thus, animals from Groups 2–5 were killed and transcardially perfused with 4% PFA maintained at 4°C, without a prior saline flush. It was found that perfusion with cold PFA dissolved the hydrogel, but left cellular graft and infiltrating cells intact.

Spinal cords from all groups were then dissected and postfixed in 4% PFA for 3 days, followed by cryoprotection in 30% sucrose (Fisher Scientific) in 0.1 M phosphate buffer pH

7.4 for 5 days, both maintained at either 37°C (Group 1) or 4°C (Groups 2–5). The spinal cords were then embedded in M-1 media (Thermo Shandon, Inc.), and fast frozen with dry ice. Tissue blocks were stored at –80°C until sliced. The spinal cord tissue blocks were cut in the horizontal plane at 20 µm thickness. Sections were collected on gelatin-coated glass slides and stored at 4°C until analyzed.

Brightfield Assessment of the Presence of the Hydrogel Within the Injury Site

Immediately after sectioning, tissue from Group 1 was imaged using a Leica DM 5500B Microscope (Leica Microsystems) with SlideBook software (Intelligent Imaging Innovations). These images allow for assessment of the lesion, as well as for identification of the hydrogel within the injury site.

Histological Assessment of the Spinal Cord Tissue

For Groups 2–5, every sixth section through the lesion was stained using Nissl and myelin to analyze injury morphology.^{12,14} Briefly, after dehydration through graded ethanols and CitroSolv (Fisher Scientific) and rehydration through graded ethanols, the slides were placed in Erichrome Cyanine R (Myelin stain), followed by differentiation in 1% aqueous ammonium hydroxide, and then stained with cresyl violet acetate (Nissl stain). Slides were again dehydrated and then coverslipped with DPX (Fluka Chemical Co.).

Fluorescent Immunohistochemical Analysis

To evaluate the presence of astrocytes, glial scar formation, host immune response, axons, and grafted cells in and around the graft site, selected sections were stained with one of the following primary antibodies: rabbit anti-GFAP for reactive astrocytes and glial scar formation; mouse anti-CSPG for glial scar formation; rabbit anti-IBA1 for reactive macrophages and microglia; mouse anti-RT-97 for host axons, graft axons, and neurofilaments; rabbit anti-CGRP for unmyelinated sensory axons; or rabbit anti-GFP for GFP-tagged proteins expressed in cells. Briefly, slides were washed in PBS with 0.2% triton X-100, and incubated in 10% goat serum (Invitrogen) in PBS for 1 hour at room temperature to block nonspecific staining. Sections were incubated in their primary antibody diluted in PBS with 2% goat serum in a humidified chamber overnight. Tissue sections were then washed in PBS, followed by incubation in the dark for 2 hours at room temperature in corresponding secondary fluorescent antibody. All secondary antibodies (Jackson Laboratories) were used at 1:400 dilution in PBS with 2% goat serum and were conjugated to either rhodamine or fluorescein isothiocyanate. Following incubation with the appropriate secondary antibodies the sections were again rinsed with PBS and coverslipped with Vectashield mounting media containing DAPI (Vector Laboratories) and stored flat at 4°C until images were obtained with the aid of a Leica DM 5500B Microscope (Leica Microsystems) with SlideBook software.

Results

Assessment of the PNIPAAm-g-PEG Implant Within the Lesion

Brightfield imaging of Groups 1a and 1b prior to staining showed that the PNIPAAm-g-PEG system gelled in situ to fill the lesion site and remained in the cavity postinjection. The

hydrogel did not degrade and appeared to act as a permanent support within the lesion. The PNIPAAm-*g*-PEG system appears to leave minor gaps, as shown only at the ventral aspect of the lesion (Fig. 1 left). In comparison, many large spaces can be identified within the cavity filled by the Gelfoam (Fig. 1 right). In the PNIPAAm-*g*-PEG system, such small gaps could be sectioning artifacts or very small cavities, whereas in the Gelfoam model or in a normal model of SCI, gaps that fill up the entire injury lesion could be the result of inflammatory activity or degradation of the Gelfoam matrix. Prior studies have also suggested that such gaps could be attributed to inflammatory activity or formation of large cavities or cysts.^{3,52} These results show the hydrogel ability to solidify in situ and fill the cavity with minor gap formation. It should be noted that images of the hydrogel within the lesion site for Group 1 could only be obtained prior to immunohistochemical and histological analysis, since the staining process resulted in loss of the thermosensitive polymer by dissolution into the aqueous medium. Brightfield images of the hydrogel in the lesion cavity for Groups 2–5 were not obtained because the hydrogel matrix was dissolved during perfusion.

Nissl and Myelin Histology

Nissl and myelin staining was performed in Groups 2–5 to determine if the hydrogels cause more demyelination in the tissue surrounding the lesion than the Vitrogen PureCol control. In Fig. 2, notice that all of the groups have similar amounts of tissue damage, indicating that the hydrogel, when seeded with a cellular transplant or with BDNF, does not cause further demyelination within the host tissue surrounding the lesion.

Inflammation and Host Immune Response

In our initial study (Group 1), we examined the host inflammatory response to PNIPAAm-*g*-PEG matrix alone at 1 week in vivo. The purpose of this initial study was to see if the scaffold alone created a significant host inflammatory response. To examine the inflammatory response to PNIPAAm-*g*-PEG, IBA-1 fluorescence immunochemistry for macrophages and microglia was performed (Fig. 3A). Results were compared directly to the host inflammatory response to Gelfoam (Fig. 3B). Both treatments appeared to have a similar presence of macrophages and microglia in the tissue surrounding the injury, indicating the expected host response to damaged tissue. Importantly, the hydrogel did not appear to elicit a greater host response than the Gelfoam.

In the subsequent study (Groups 2–5), IBA-1 staining was performed after 2 weeks in vivo on PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC scaffolds in combination with a cellular graft or with BDNF. As previously mentioned, animals in Group 2, containing PNIPAAm-*g*-PEG and the cell graft, received treatment with the cyclosporin A immunosuppressant, which reduces T cells and reduces the inflammatory response.^{17,18,32} The responses were compared with Vitrogen PureCol with BDNF. The IBA-1 staining in Fig. 3C–F highlights the reactive macrophages and microglia present in the tissue around and within the lesion. We observed that the synthetic PNIPAAm-*g*-PEG hydrogel (Group 3, Fig. 3D), the semisynthetic PNIPAAm-*g*-MC hydrogel (Group 4, Fig. 3E), and the control group (Group 4, Fig. 3F) elicited comparable host inflammatory responses. We also observed that the 3 previously mentioned groups elicited comparable host inflammatory responses to the PNIPAAm-*g*-

PEG/RSF cell graft group (Group 2, Fig. 3C), indicating that the scaffold itself, without immune suppression, does not appear to induce a greater immune response than is seen in the group receiving immune suppression. DAPI staining shows that cells are migrating from the host tissue into the lesion site, suggesting that PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC, like PureCol and Gelfoam, are permissive to cell migration and infiltration (Fig. 3A, B, D, and F). All of the lesions show the presence of inflammatory cells within the injury site, as seen in Fig. 3C–F. The presence of inflammatory cells seen within the lesion in the PNIPAAm-*g*-PEG/RSF cell graft group (Group 2, Fig. 3C) can at least in part be attributed to the host immune system recognizing the grafted cells as foreign.

Identification of RSF Grafts Within the Lesion Cavity

Rat fibroblasts infected with a lentiviral construct containing the GFP reporter gene and BDNF (RSF-GFP BDNF) were grafted into the injury lesion. Fibroblast grafts can be identified easily by the presence of GFP-expressing cells in the lesion area. The GFP-positive cells are seen in the lesion cavity (Fig. 4 lower). This shows that PNIPAAm-*g*-PEG can deliver cellular grafts to a lesion area and that it can support cell survival. Importantly, these results indicate that grafted cells remain in the hydrogel and do not immediately leave the matrix.

Glial Scar Formation

We next determined if the PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC systems caused more glial scar formation than commercially available matrices. Glial scar formation was assessed with primary antibodies for detection of GFAP and CS-56 CSPG. The GFAP antibody stains astrocytes that can become reactive and create a physical boundary to regenerating axons and their targets.^{20,28,46} Chondroitin sulfate proteoglycans have been shown to be closely associated with neuronal growth inhibition⁴¹ and are upregulated in the CNS after injury, specifically around the lesion site where the glial scar forms. In Fig. 5, the center column shows GFAP staining for Groups 2–5. All groups, including the Vitrogen PureCol control, show a comparable presence of astrocytes around the lesions. Reactive astrocytes, rather than just creating a barrier around the injury site, were present within the lesion in all of the groups. The images in the right column of Fig. 5 show CS-56 staining, which is also a marker of the glial scar. Again, we see comparable CSPG staining around the lesions in Groups 2–5. Our results indicate that PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC do not contribute to more glial scar formation compared with the control matrix, Vitrogen PureCol.

Axonal Growth

RT-97 staining highlights neurofilaments and individual axons. It is necessary that a scaffold for neural regeneration be permissive to axonal growth. Figure 6 shows that both the hydrogel and Vitrogen PureCol, when combined with BDNF-expressing fibroblasts or BDNF (Groups 2–5), promote neurofilament growth within the injury site (center column). Our results also suggest that PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC are permissive to axonal growth.

Figure 6 (right column) also shows tissue sections stained with CGRP, which labels a subpopulation of sensory axons. This allows identification of axons regenerating specifically

from the dorsal root placed next to the cavity. In the hydrogel groups (Groups 2–4) and in the Vitrogen PureCol group (Group 5), we observed sensory axons from the dorsal root growing into the matrix within the lesion cavity. These results suggest that a dorsal root placed next to the lesion during surgery can regenerate axons into the graft.

Discussion

PNIPAAm-g-PEG and PNIPAAm-g-MC are Feasible Injectable Scaffolds for SCI

Scaffold-based approaches for SCI involve the use of a polymeric material to fill the lesion site, but many of these polymeric materials are composed of biodegradable polyesters.^{5,8,26,31,39} While such materials exhibit good biocompatibility, their implantation into irregularly shaped defects is difficult, since they are preformed. Given that the currently proposed PNIPAAm-based materials are injectable, they are much easier to implant. In our in vivo work, the PNIPAAm-*g*-PEG scaffolds were successfully injected as viscous liquids during surgery and transitioned to solid gels at body temperature. Brightfield analysis of the lesion site (Group 1) prior to staining showed that the PNIPAAm-*g*-PEG hydrogel is completely space filling, suggesting that it can function as a matrix for regenerating axons. Our prior work showed that PNIPAAm-*g*-PEG is nondegradable,⁵¹ so it is expected that these materials will provide permanent support throughout the regeneration process. It should be noted that while in situ gelation of the PNIPAAm-*g*-MC was successful, 8 wt% PNIPAAm-*g*-MC is much more viscous than 10 wt% PNIPAAm-*g*-PEG in water at room temperature. Since both hydrogels exhibited comparable in vivo performance here, PNIPAAm-*g*-PEG may be considered the more favorable material because it would allow for easier, more uniform suspension of cells due to the lower viscosity. As noted before, Brightfield images of the PNIPAAm-*g*-MC-filled lesion sites could not be taken because the thermosensitive hydrogels were washed away during perfusion with cold PFA (Groups 2–5). In future studies, perfusion with warm saline or PFA at the time the rats are killed would allow for retention of the thermosensitive hydrogel within the spinal cord defect and allow for examination of any possible degradation of PNIPAAm gels that may occur with the incorporation of natural biopolymer MC.

PNIPAAm-g-PEG Supports Delivery and Survival of Cells In Vivo

Our prior published work showed that PNIPAAm-*g*-PEG scaffolds are capable of supporting mesenchymal stem cell attachment and viability in vitro.⁷ Our additional unpublished in vitro studies with RSF-GFP BDNF-expressing fibroblasts further confirmed the ability of both PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC to support cell attachment and survival (results not shown). In the in vivo studies described here, the same rat fibroblasts were successfully mixed with the PNIPAAm-*g*-PEG below its LCST and delivered to the injury site. The graft was identified using GFP fluorescence, since the fibroblasts were infected with a lentiviral construct containing the GFP reporter gene and BDNF. Successful identification of the graft within the lesion suggests that PNIPAAm-*g*-PEG can deliver rat fibroblast cellular grafts to a lesion area without migration out of the cavity and that it supports rat fibroblast cell survival. Although PNIPAAm-*g*-MC was not studied for cell delivery, we expect similar results. As noted before, the higher viscosity of PNIPAAm-*g*-MC solutions could make uniform cell suspension more difficult.

PNIPAAm-g-PEG and PNIPAAm-g-MC Elicit Comparable Inflammatory Responses From the Host Tissue

Inflammatory response is the process by which macrophages attempt to phagocytose foreign material and encapsulate it in a protective layer in an attempt to seal it off from healthy tissue. Microglia rid the CNS of damaged neurons, plaque, and other infectious agents.¹⁰ In this study, it was important to assess the effect of the scaffolds on the host immune response. Using IBA-1 staining for macrophages and microglia in our preliminary experiment, we observed that the PNIPAAm-*g*-PEG hydrogel by itself elicited an inflammatory response similar to that of the commercially available Gelfoam matrix, which shows the biocompatibility of this scaffold material. In our later study, we again observed a comparable host response to the hydrogel groups loaded with BDNF, when compared with the Vitrogen PureCol/BDNF group. Our results from this staining indicate that PNIPAAm-*g*-PEG/BDNF and PNIPAAm-*g*-MC/BDNF do not elicit greater immune responses than the commercially available collagen matrix. Furthermore, the use of semisynthetic versus completely synthetic PNIPAAm-based hydrogels had no observable effect on the host response.

As can be seen from Fig. 3, all of the lesions show the presence of inflammatory cells within the injury site. While some studies have shown that after SCI, infiltration of activated macrophages correlates with long-distance retraction of dystrophic end bulbs, known as axonal dieback,¹⁶ not all researchers agree that macrophages inhibit neuronal regeneration and axonal growth. Hirschberg et al.¹⁵ concluded that inflammation after axonal injury has dual effects. Their results showed that while rescue of spared axons is impaired, regeneration is supported. Schwartz et al.⁴² showed that the implantation of prestimulated, autologous macrophages into completely transected spinal cords of adult rats led to partial motor recovery. In addition, Prewitt et al.⁴⁰ concluded that increasing the presence of activated macrophage/microglial cells at an SCI site can provide an environment beneficial to the promotion of regeneration of sensory axons, possibly by the release of cytokines and interaction with other nonneuronal cells in the immediate vicinity. In our study, if we examine the neurofilament staining for PNIPAAm-*g*-PEG/RSF cell suspension (Group 2) in Fig. 6B, our results show the presence of neurofilaments and axonal growth into the cavity. This indicates that the presence of inflammatory cells does not appear to inhibit axonal growth into the cavity. While the aim of this paper is to determine the feasibility of the scaffolds relative to the current industry standard, specifically Gelfoam or Vitrogen PureCol, it should be noted that any implanted scaffold affects the postinjury environment and host response, in comparison with a scaffold-free postinjury environment.

Glial Scar Formation is Not Affected by PNIPAAm-g-PEG and PNIPAAm-g-MC

Another host response at the injury site is the formation of the glial scar, created mainly by reactive astrocytes and microglia, isolating healthy tissue from the injury and creating a boundary to reconnection of axons with their targets. The PNIPAAm copolymer groups do not show reactive astrocytes infiltrating deep into the lesions, and they do not lead to increased formation of the glial scar around the injury sites. This indicates that the PNIPAAm-*g*-MC and PNIPAAm-PEG do not augment astrocyte reactivity and glial scar formation more than the commercially available matrix. Again, the use of semisynthetic

versus completely synthetic PNIPAAm-based hydrogels had no observable effect on scar formation.

PNIPAAm-g-PEG and PNIPAAm-g-MC are Permissive to Axon Growth

An effective scaffold must be permissive to axon growth. The goal of this particular work was to assess if the scaffold allows axon growth within the lesion through the action of BDNF. We also aimed to determine whether incorporation of natural polymer with established biocompatibility would render our injectable matrix more permissive to axonal growth. To better assess the bioactivity and the expected release profile of BDNF from a scaffold, in vitro work by Comolli et al.⁷ previously determined that codissolved BDNF is released in bioactive form from PNIPAAm-*g*-PEG hydrogels with a minimal burst, followed by a linear release for 4 weeks. Neurite outgrowth assays using chick embryo dorsal root ganglion showed that the released BDNF remained biologically active after 31 days in the PNIPAAm-*g*-PEG scaffold.

RT-97 staining for neurofilaments, within lesions filled with PNIPAAm-*g*-PEG loaded with fibroblasts, PNIPAAm-*g*-PEG loaded with BDNF, and PNIPAAm-*g*-MC loaded with BDNF shows that all matrices are permissive to host axon growth. To label a subpopulation of sensory axons specifically from the dorsal root, CGRP staining was performed. In all of the hydrogel groups, we identified CGRP-positive sensory axons growing from the dorsal root into the grafted matrix within the lesions. Although quantification was not performed, results show that incorporation of the MC into the PNIPAAm network did not appear to increase axonal growth compared with PEG. These results indicate that host axonal growth into a scaffold is dependent on other factors, such as porosity and presence of trophic factors, rather than chemical composition.

Since our sample groups were small (3 rats per group), we did not have the statistical power to precisely quantify our preliminary data; however, our main goal in these pilot studies was to show that the hydrogels are feasible materials to use in future studies involving SCI and recovery of function. Now that we have shown that the hydrogels are permissive to axon growth, larger studies will be performed to quantify the distance of axon growth achieved, as well as axon-tracing studies to determine the source of the host-derived axons or sensory axons present within the graft. Future work will also investigate the simultaneous delivery of cells and neurotrophic factors to SCI with the multifunctional PNIPAAm-based scaffolds. Specifically, we will investigate the use of lineage-restricted neural precursor cells, which were previously shown to survive and differentiate into neural cells in the injured CNS and also to provide a permissive niche for axonal regeneration.^{25,33}

Conclusions

This study used two established models of SCI to demonstrate the feasibility of PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC injectable scaffolds as treatment options for SCI by evaluating their ability to support cell survival and axonal growth without further contributing to the host immune response. The ability of the hydrogels to be injected as viscous liquids allows for easy implantation into the cavity. We demonstrated that the thermosensitive nature of the hydrogels also allows them to form space-filling gels within a spinal cord lesion. Moreover,

results suggest that PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC, in combination with BDNF, are permissive to host axon growth, shown by neurofilament staining within the lesion. The hydrogels also do not elicit greater host inflammatory responses or scar formation than commercially available matrices. In addition, PNIPAAm-*g*-PEG was shown to successfully deliver cellular grafts in vivo. Although the in vivo performances of the PNIPAAm-*g*-PEG and PNIPAAm-*g*-MC scaffolds were comparable, the lower viscosity of the PNIPAAm-*g*-PEG at room temperature makes it easier to use for the treatment of SCI in combination with cellular grafts.

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Abbreviations used in this paper

BDNF	brain-derived neurotrophic factor
CGRP	calcitonin gene-related peptide
CSPG	chondroitin sulfate proteoglycan
LCST	lower critical solution temperature
MA	methacrylic anhydride
MC	methylcellulose
NIPAAm	<i>N</i> -isopropylacrylamide
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PFA	paraformaldehyde
PNIPAAm	poly(<i>N</i> -isopropylacrylamide)
RSF	rat skin fibroblast
SCI	spinal cord injury

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Fig. 1.

Brightfield images of injury cavities 1 week after grafting. Grafts are outlined by *boxes*.

Left: Brightfield image of PNIPAAm-*g*-PEG (Group 1a) showing that the polymer gel fills the cavity with minimal gap formation. **Right:** In comparison, the Gelfoam implant (Group 1b) has many large spaces within the cavity. Bar = 200 μ m.

Fig. 2.

Hydrogels do not cause additional demyelination in adjacent tissue. Myelin staining (*blue*) highlights the intact white matter (WM) of the spinal cord. **A–H:** Demyelination is only present at the injury site, not in surrounding tissue. Low- (**A**) and high- (**B**) magnification PNIPAAm-*g*-PEG/RSF cell suspension (Group 2). Low- (**C**) and high- (**D**) magnification PNIPAAm-*g*-PEG/BDNF (Group 3). Low- (**E**) and high- (**F**) magnification PNIPAAm-*g*-MC/BDNF (Group 4). Low- (**G**) and high- (**H**) magnification Vitrogen PureCol/BDNF (Group 5). Bar = 500 μ m. GM = gray matter.

Fig. 3.

Hydrogels do not elicit a greater host response than is seen in the control groups. IBA-1 (*red*) staining was used to assess the presence of reactive macrophages and microglia adjacent to the lesion and in the surrounding host tissue. DAPI labeling (*blue*) was used to identify cell nuclei. **A:** Immunohistochemical labeling of macrophages and microglia, rostral to a graft of PNIPAAm-*g*-PEG (Group 1a). **B:** IBA-1 labeling rostral to a graft of Gelfoam (Group 1b). **C:** PNIPAAm-*g*-PEG/RSF cell suspension (Group 2). **D:** PNIPAAm-*g*-PEG/BDNF (Group 3). **E:** PNIPAAm-*g*-MC/BDNF (Group 4). **F:** Vitrogen PureCol/BDNF (Group 5). Bar = 200 μ m.

Fig. 4. Cellular grafts were successfully delivered to the lesion site by encapsulation within the injectable gels. **Upper:** Low-magnification Nissl and myelin-stained image to help in evaluating the lesion size and the location of the lesion within the host tissue. DAPI labeling (*blue*) was used to identify cell nuclei. **Lower:** The rat fibroblast graft within the lesion (PNIPAAm-*g*-PEG/RSF cell suspension, Group 2) is identified using the GFP fluorescent marker (*green*). Bars = 500 μ m (**upper**); 200 μ m (**lower**).

Fig. 5.

Hydrogels do not contribute to more glial scar formation than the control matrix. Low-magnification Nissl and myelin-stained images obtained in each group to help in evaluating the lesion size and the location of the lesion within the host tissue (*left column*). GFAP (*green, center column*) and CS-56 (*red, right column*) staining of astrocytes and chondroitin sulfate proteoglycans around graft site. DAPI labeling (*blue*) was used to identify cell nuclei. **A–C:** PNIPAAm-*g*-PEG/RSF cell suspension (Group 2). **D–F:** PNIPAAm-*g*-PEG/BDNF (Group 3). **G–I:** PNIPAAm-*g*-MC/BDNF (Group 4). **J and K:** Vitrogen PureCol/BDNF (Group 5). **L:** Vitrogen PureCol/BDNF (Group 5), dorsal to the injury site. Bar = 500 μ m (**A, D, G, and J**); 200 μ m (**B, C, E, F, H, I, and K**); 100 μ m (**L**).

Fig. 6.

Hydrogels are permissive to axonal growth. Lesion sites were stained for neurofilaments and CGRP. Low-magnification Nissl and myelin-stained images from each group to help in evaluating the lesion size and the location of the lesion within the host tissue (*left column*). RT-97 (*red*) stains host axons (*center column*), and CGRP (*red*) labels sensory axons. DAPI labeling (*blue*) was used to identify cell nuclei (*right column*). In all CGRP images, notice the dorsal root next to the injury site, shown with an *arrow*. **A–C:** PNIPAAm-*g*-PEG/RSF cell suspension (Group 2). **D–F:** PNIPAAm-*g*-PEG/BDNF (Group 3). **G–I:** PNIPAAm-*g*-MC/BDNF (Group 4). **J–L:** Vitrogen PureCol/BDNF (Group 5). Bar = 500 μ m (**A, D, G, and J**); 200 μ m (**B, C, E, F, H, I, K, and L**).

TABLE 1

Lesion site implantations

Group No.	No. of Rats	Implant	Survival (wks)	Perfusion Chemicals & Temperature
1a	3	3 μ l 10 wt% PNIPAAm-PEG	1	0.9% saline & 4% PFA, both at 37°C
1b	3	1 piece of Gelfoam sponge	1	0.9% saline & 4% PFA, both at 37°C
2	3	6 μ l 10 wt% PNIPAAm- <i>g</i> -PEG loaded w/ 5.0×10^4 cells/ μ l of RSF-GFP BDNF cell suspension	2	Only 4% PFA at 4°C
3	3	6 μ l 10 wt% PNIPAAm- <i>g</i> -PEG loaded w/ 0.05 μ g/ μ l BDNF	2	Only 4% PFA at 4°C
4	3	6 μ l 8 wt% PNIPAAm- <i>g</i> -MC loaded w/ 0.05 μ g/ μ l BDNF	2	Only 4% PFA at 4°C
5	3	6 μ l Vitrogen PureCol loaded w/ 0.05 μ g/ μ l BDNF	2	Only 4% PFA at 4°C