

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
17 January 2008 (17.01.2008)

PCT

(10) International Publication Number
WO 2008/008288 A2

(51) International Patent Classification:
A61K 35/30 (2006.01)

(74) Agent: **ROCHE, Richard, T.**; Quarles & Brady LLP, 411 E. Winsconsin Ave., Suite 2040, Milwaukee, WI 53202 (US).

(21) International Application Number:

PCT/US2007/015612

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(22) International Filing Date: 6 July 2007 (06.07.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/819,846 11 July 2006 (11.07.2006) US

(71) Applicant (for all designated States except US): **MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH** [US/US]; 200 First Street SE, Rochester, MN 55905 (US).

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

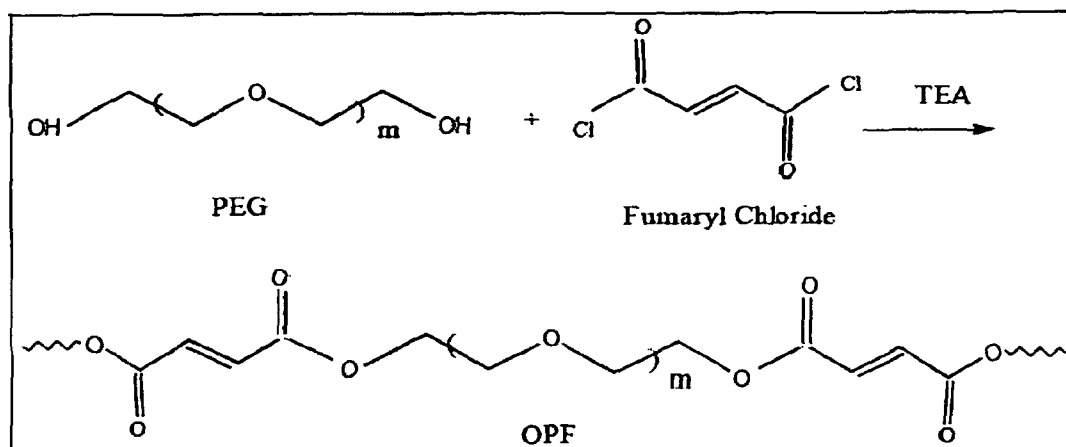
(75) Inventors/Applicants (for US only): **DADSENTAN, Mahrokh** [IR/US]; 835 40th Street NW #A210, Rochester, MN 55901 (US). **YASZEMSKI, Michael, J.** [US/US]; 2806 15th Ave. SW, Rochester, MN 55902 (US). **LU, Lichun** [CN/US]; 734 27th St. NW, Rochester, MN 55901 (US).

Published:

— without international search report and to be republished upon receipt of that report

[Continued on next page]

(54) Title: CHARGED OLIGO(POLY(ETHYLENE GLYCOL) FUMARATE) HYDROGELS FOR NERVE GROWTH



(57) Abstract: A biodegradable material for improving the regeneration of nerve cells is disclosed. The material includes a copolymer formed by reacting a first reactant selected from monomers, oligomers and polymers and a second charged reactant selected from charged monomers, charged oligomers and charged polymers. Nerve cells are contained within or attracted to the copolymer. The first reactant can be oligo(poly(ethylene glycol) fumarate). The charged reactant can be selected from unsaturated quaternary ammonium compounds. The material can include a photoinitiator such that the material is photocrosslinkable. The material can include a bioactive agent such as a nerve growth factor. In one form, the material is a hydrogel that can be injected as a fluid into a patient's body via minimally invasive arthroscopic techniques to form a scaffold for nerve tissue regeneration.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Charged Oligo(poly(ethylene glycol) fumarate) Hydrogels for Nerve Growth

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority from United States Provisional Patent Application No. 60/819,846 filed July 11, 2006.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under grant numbers R01-AR45871 and EB02390 and R01-EB003060 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0003] This invention relates to a biodegradable material for improving the regeneration of nerve cells. The material includes a copolymer formed by reacting a first reactant selected from monomers, oligomers and polymers, and a second reactant selected from charged monomers, charged oligomers and charged polymers. Nerve cells are contained within or attracted to the copolymer. In one example, copolymers formed from an oligo(poly(ethylene glycol) fumarate) hydrogel and a quaternary ammonium compound and including nerve cells have been shown to improve neurite outgrowth.

2. Description of the Related Art

[0004] The current clinical approach to repair segmented peripheral nerve defects involves utilization of an autologous nerve graft. However, autografts have several drawbacks including loss of function in the donor sensory nerve graft site and size mismatch of damaged nerve. As an alternative to nerve autografts, a number of different natural and synthetic materials have been explored for use in aiding nerve regeneration (see, Schmidt *et al.*, (2003) *Annu Rev Biomed Eng* 5, 293-347). Although natural materials have inherent bioactivity and biocompatibility that may aid in nerve regeneration, synthetic materials offer several advantages such as controllable physical and biochemical properties that can be tailored for specific application. In addition, degradation rates of these materials can be easily controlled for the final use. Poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and poly(lactic-acid-coglycolic acid) (PLGA) were some of the first synthetic polymers studied because of their availability, ease of processing, biodegradation, and FDA approval (see, Molander *et al.*, (1982)

Muscle Nerve 5, 54-7; and Evans *et al.*, (2000) *J Biomater Sci Polym Ed* 11, 869-78; and Hurtado *et al.*, (2006) *Biomaterials* 27, 430-42). Several nondegradable polymers have been also used in nerve repair applications, including silicone tubing and expanded poly(tetrafluoroethylene) (see, Dahlin *et al.*, (2001) *J Hand Surg [Br]* 26, 393-4; and Vasconcelos *et al.*, (2000) *J Oral Maxillofac Surg* 58, 1257-62). Silicone, in particular, has been studied as a model system for nerve regeneration since the 1960s. However, it is impermeable and inert such that guidance channels do not support regeneration across defects larger than 10 millimeters (in rat models) without the presence of exogenous growth factors (see, Ducker *et al.*, (1968) *J Neurosurg* 28, 582-7).

[0005] Currently, attempts are being made to develop semi permeable or degradable guidance channels that can actively stimulate nerve regeneration over longer more clinically relevant defect lengths. See, for example, U.S. Patent No. 7,163,545. Both degradable and nondegradable hydrogels have been applied to nerve regeneration applications due to their permeability and biocompatibility (see, Burdick *et al.*, (2006) *Biomaterials* 27, 452-9; and Gunn *et al.*, (2005) *J Biomed Mater Res A* 72, 91-7; and Bellamkonda *et al.*, (1995) *J Biomed Mater Res* 29, 663-71). The hydrogels are made up of a water-saturated network which helps maintain a physiological environment in the implantation site that is suitable for the diffusion of trophic molecules released from the reactive tissue bordering the lesion after transection. In one approach, polyethylene glycol (PEG) solution combined with a crosslinkable PEG-based hydrogel have been used to fuse the membranes of severed nerve ends of sciatic and spinal nerves (see, Lore *et al.*, (1999) *J Neurosci* 19, 2442-54). These studies have shown that conduction of axon potentials can be restored immediately with this procedure. However, this process is only applicable if the severed nerve ends are adjacent, and therefore cannot be used for large nerve defects. Among other hydrogels, PEG-based hydrogels have been extensively studied for their use in tissue engineering and regenerative medicine applications (see, Burdick *et al.*, (2006) *Biomaterials* 27, 452-9; and Collier *et al.*, (2000) *J Biomed Mater Res* 50, 574-84; and Bellamkonda *et al.*, (1995) *J Neurosci Res* 41, 501-9). The drawback for PEG-based hydrogels is a low cell attachment to their surfaces resulting from formation a hydrated layer on their surfaces that inhibits adsorption of adhesion-specific

proteins such as fibronectin. Recent studies are focused on modification of these systems to improve cell attachment to their surfaces. Researchers have shown that PC12 cells are able to extend neurites on crosslinked PEG hydrogels when the cell adhesion peptide RGDS is incorporated into the materials (see, Gunn *et al.*, (2005) *J Biomed Mater Res A* 72, 91-7). Other investigators reported enhanced osteoblasts and fibroblasts attachment to the PEG-based and HEMA hydrogels with incorporation of positively and negatively charged monomer (see, Schneider *et al.*, (2004) *Biomaterials* 25, 3023-8; and Sosnik *et al.*, (2005) *J Biomed Mater Res A* 75, 295-307). However, the effects of charge incorporation into these hydrogels on nerve cells attachment and neurite extension have not been studied.

[0006] Furthermore, other researchers have shown that electrical charges play an important role in stimulating either the proliferation or differentiation of various cell types. Neurite extension, for example, is significantly enhanced on piezoelectric materials (i.e., materials that generate a surface charge with small deformations), such as poly(vinylidene fluoride) and on electrically conducting polymers, such as poly(pyrrole). They have demonstrated that neurite outgrowth is enhanced on positively charged fluorinated ethylene propylene electret films in both serum free and serum containing media compared to negative and uncharged films and neurite outgrowth is highly correlated to the magnitude and polarity of the charge (see, Makohliso *et al.*, (1993) *J Biomed Mater Res* 27, 1075-85). Other investigators have also shown enhancing effect of polycationic chitosan on embryonic chick dorsal root ganglia neurite extension (see, Dillon *et al.*, (2000) *J Biomed Mater Res* 51, 510-9). Other investigators have demonstrated that electrical charges play an important role in stimulating either the proliferation or differentiation of various cell types, and enhanced neurite outgrowth has been seen on electrets, piezoelectric and conductive polymers (see, Schmidt *et al.*, PNAS, 94, 8984, 1997; Arundhati Hotwal *et al.*, Biomaterials 22, 1055, 2001; and U.S. Patent Nos. 6,696,575 and 6,095,148). However, these materials are generally not degradable and their biocompatibility has not been studied extensively.

[0007] Recently, natural and synthetic hydrogels have attracted much attention for nerve regeneration, as they are made up of 90% water and their mechanical

properties closely match with those of soft nerve tissue. In addition, growth factors can be incorporated into the hydrogels to direct nerve regeneration. Hydrogels are highly biocompatible and biodegradable hydrogels that are mostly water and can be readily absorbed by the body. Oligo (poly (ethylene glycol) fumarate) (OPF) is one macromer that has been developed and has been used for fabrication of hydrogels with a redox initiation system. It is reported that OPF hydrogels are biodegradable and their mechanical properties and degradation rates are controlled by the molecular weight of the PEG used in macromer formation. (See, Jo *et al.*, "Modification of oligo(poly(ethylene glycol) fumarate) macromer with a GRGD peptide for the preparation of functionalized polymer networks", *Biomacromolecules* 2001;2(1):255-61 ; and Temenoff *et al.*, "Effect of poly(ethylene glycol) molecular weight on tensile and swelling properties of oligo(poly(ethylene glycol) fumarate) hydrogels for cartilage tissue engineering", *J Biomed Mater Res* 2002;59(3):429-37; and U.S. Patent No. 6,884,778; and U.S. Patent Application Publication No. 2002/0028189.)

[0008] However, there is still a need for a biodegradable material for improving the regeneration of nerve cells.

SUMMARY OF THE INVENTION

[0009] The present invention provides a biodegradable material for improving the regeneration of nerve cells. The material includes a biocompatible, biodegradable copolymer formed by reacting a first reactant selected from monomers, oligomers and polymers and a second reactant selected from charged monomers, charged oligomers and charged polymers. Nerve cells are contained within or attracted to the copolymer. The material may include a photoinitiator such that the material is photocrosslinkable. The material may include a bioactive agent such as a nerve growth factor. The material may include myelinating cells such as Schwann cells or oligodendrocytes. In one form, the material is a hydrogel that can be injected as a fluid into a patient's body via minimally invasive arthroscopic techniques to form a scaffold for nerve tissue regeneration. An important characteristic of the biodegradable materials is that they crosslink in a few minutes at room temperature using low power UV light and a cytocompatible photoinitiator. These hydrogels have a high degree of swelling in aqueous environments, and can maintain their structural integrity at water contents above

95%. Thus, they can be applied for nerve cell encapsulation and support nerve cell viability in constructs that are several millimeters thick, since the exchange of nutrients and wastes can occur over distances of this magnitude in water. Additionally, the synthetic matrix properties (e.g. crosslink density, water content, modulus, and surface tension) can be tailored to fit its end use. In one example form, the first reactant is oligo(poly(ethylene glycol) fumarate), the charged reactant is selected from unsaturated quaternary ammonium compounds, and a weight ratio of oligo(poly(ethylene glycol) fumarate) to the charged reactant in the material is in the range of 1:0.01 to 1:0.5.

[0010] In the biodegradable material, the nerve cells can be neurons. The charged reactant can be cationic. The charged reactant can be selected from quaternary ammonium compounds such as quaternary ammonium salts or quaternary ammonium halides. The charged reactant can be also selected from acrylate and methacrylate monomers. The material can include a photoinitiator such that the material is photocrosslinkable, preferably in the temperature range of 30°C to 45°C. The material can be injectable. The biodegradable material can include a porogen to create a porous material upon crosslinking.

[0011] In one aspect, the invention provides a biodegradable material including (i) a hydrogel prepared by reacting oligo(poly(ethylene glycol) fumarate) and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers; and (ii) nerve cells contained within or attracted to the hydrogel. In one form, the hydrogel comprises 95 weight percent or more water. The nerve cells can be neurons, and the hydrogel can include a bioactive agent, such as a nerve growth factor, and/or myelinating cells. The hydrogel can be photocrosslinkable in an aqueous solution. In one form, the charged reactant is selected from quaternary ammonium compounds, or from acrylate and methacrylate monomers, or from acrylate and methacrylate quaternary ammonium monomers.

[0012] In another aspect, the invention provides a scaffold for nerve tissue regeneration. The scaffold includes (i) a biodegradable matrix prepared by reacting oligo(poly(ethylene glycol) fumarate) and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers; and (ii) nerve cells contained within or attracted to the matrix. The

oligo(poly(ethylene glycol) fumarate) can be provided as a hydrogel. The matrix can be prepared by photocrosslinking the oligo(poly(ethylene glycol) fumarate) and the charged reactant in the presence of a photoinitiator. In one version, the nerve cells are encapsulated in the matrix. In another version, the nerve cells are adhered to a surface of the matrix. The scaffold can include myelinating cells. The matrix can be prepared by crosslinking the oligo(poly(ethylene glycol) fumarate) and the charged reactant in the presence of a porogen. In one version, the scaffold has a porosity of 70% to 90%. The scaffold can be a nerve conduit.

[0013] In yet another aspect, the invention provides a method for nerve tissue regeneration. In the method, a material including (i) a copolymer formed by reacting a first reactant selected from the group consisting of monomers, oligomers and polymers and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers, and (ii) nerve cells is injected into a location in a patient's body; and the material is crosslinked, optionally in the presence of a porogen. The material can include a photoinitiator, and the step of crosslinking can include photocrosslinking the material. In one form, the first reactant is oligo(poly(ethylene glycol) fumarate) provided as a hydrogel, and the charged reactant is selected from quaternary ammonium compounds, or from acrylate and methacrylate monomers, or from acrylate and methacrylate quaternary ammonium monomers. The nerve cells can be neurons, and the material can include myelinating cells.

[0014] In still another aspect, the invention provides a method for nerve tissue regeneration. In the method, a scaffold including (i) a biodegradable matrix including a copolymer formed by reacting a first reactant selected from the group consisting of monomers, oligomers and polymers and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers, and (ii) nerve cells contained within or attracted to the matrix is implanted into a patient's body for nerve tissue regeneration. In one form, the first reactant is oligo(poly(ethylene glycol) fumarate) provided as a hydrogel, and the charged reactant is selected from quaternary ammonium compounds, or from acrylate and methacrylate monomers, or from acrylate and methacrylate quaternary ammonium monomers. The nerve cells can be neurons, and the material can include myelinating cells.

[0015] These and other features, aspects, and advantages of the present invention will become better understood upon consideration of the following detailed description, drawings, and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] Figure 1 shows the synthesis of oligo(poly(ethylene glycol) fumarate) (OPF) from polyethylene glycol and fumaryl chloride.

[0017] Figure 2 shows a schematic of a photocrosslinking reaction for the fabrication of oligo(poly(ethylene glycol) fumarate) (OPF) hydrogels.

[0018] Figure 3 shows the swelling ratios of the positively charged OPF hydrogels with different concentrations of a charged monomer (MAETAC).

[0019] Figure 4 shows the compression modulus of the positively charged OPF hydrogels with different concentrations of a charged monomer (MAETAC).

[0020] Figure 5A shows PC12 Cells on an OPF hydrogel without a charged monomer.

[0021] Figure 5B shows PC12 Cells on an OPF hydrogel with a charged monomer.

[0022] Figure 5C shows GFP Schwann cells on an OPF hydrogel with a charged monomer.

[0023] Figure 6 shows two types of dorsal root ganglion (DRG) cell cultures used in Example 1.

[0024] Figure 7 shows dissociated dorsal root ganglion cell attachment and neurite outgrowth on charged modified hydrogels after 7 days.

[0025] Figure 8 shows dorsal root ganglion explant and neurite outgrowth on positively charged hydrogels after 40 hours.

[0026] Figure 9 shows dorsal root ganglion explant neurite lengths on positively charged hydrogels after 24 and 40 hours.

[0027] Figure 10 shows dorsal root ganglion explant neurite lengths on positively charged hydrogels after 40 Hours.

[0028] Figure 11 shows the frequency of neurite outgrowth for dorsal root ganglion explant grown on charged hydrogels.

[0029] Figure 12 shows the myelination of axons on charged hydrogel after 3 weeks.

[0030] Figure 13 shows an OPF multi-lumen nerve guide fabricated with a stereolithography machine. It is a prototype of a 19 channel OPF-Hydrogel nerve conduit.

[0031] Figure 14 shows Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) data of unmodified and modified OPF hydrogels.

[0032] Figure 15 shows ATR-FTIR data of unmodified and modified OPF hydrogels.

[0033] Figure 16 shows Young's modulus of hydrogels with different formulations.

DETAILED DESCRIPTION OF THE INVENTION

[0034] The invention provides a biodegradable material for improving the regeneration of nerve cells. The material includes a copolymer formed by reacting (i) a first reactant selected from the group consisting of monomers, oligomers and polymers, and (ii) a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers. Nerve cells are contained within or may be attracted to (i.e., chemically bonded to) the copolymer. Preferably, the weight ratio of the first reactant to the charged reactant in the material is in the range of 1:0.01 to 1:0.5. The first reactant may be oligo(poly(ethylene glycol) fumarate). As used herein, a "biodegradable" material is one which decomposes under normal in vivo physiological conditions into components which can be metabolized or excreted; and a "biocompatible" material is one which stimulates only a mild, often transient, implantation response, as opposed to a severe or escalating response.

[0035] The charged reactant may be cationic, and preferably the charged reactant is selected from the group consisting of unsaturated quaternary ammonium compounds, and most preferably the charged reactant is selected from the group consisting of unsaturated quaternary ammonium salts. The charged reactant may be selected from acrylate and methacrylate monomers, or from acrylate and methacrylate quaternary ammonium monomers. Non-limiting examples of the charged reactant include quaternary ammonium halides such as [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC).

[0036] The material may include a photoinitiator such that the material is photocrosslinkable. Preferably, the material photocrosslinks in the temperature range of 30°C to 45°C. By "photocrosslinkable", we mean the functional groups of a polymer may crosslink with the functional groups of the same polymer or another monomer or polymer by application of photons (e.g., UV light) in the presence of a photoinitiator. Preferably the material is injectable. By "injectable", we mean the material may be delivered to a site by way of a medical syringe or an arthroscopic device. The material may include a porogen to create a porous structure upon crosslinking. Non-limiting examples of porogens include solid salts and hydrogel particles.

[0037] The material may include a bioactive agent. A "bioactive agent" as used herein includes, without limitation, physiologically or pharmacologically active substances that act locally or systemically in the body. A bioactive agent is a substance used for the treatment, prevention, diagnosis, cure or mitigation of disease or illness, or a substance which affects the structure or function of the body or which becomes biologically active or more active after it has been placed in a predetermined physiological environment. Bioactive agents include, without limitation, enzymes, organic catalysts, ribozymes, organometallics, proteins, glycoproteins, peptides, polyamino acids, antibodies, nucleic acids, steroidal molecules, antibiotics, antimycotics, cytokines, growth factors (e.g., nerve growth factors), carbohydrates, oleophobic, lipids, extracellular matrix and/or its individual components, pharmaceuticals, and therapeutics. Preferably, the bioactive agent is a nerve growth factor.

[0038] In one form, the material is a biodegradable hydrogel prepared by reacting oligo(poly(ethylene glycol) fumarate) and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers, and nerve cells are contained within or attracted to the hydrogel. The hydrogel may comprise 95 weight percent or more water. The hydrogel may include a bioactive agent such as a nerve growth factor. The hydrogel is photocrosslinkable in an aqueous solution. In one example, the charged reactant is selected from the group consisting of unsaturated quaternary ammonium compounds.

[0039] In another form, the invention provides a scaffold for nerve tissue regeneration. The scaffold includes a biodegradable matrix prepared by reacting oligo(poly(ethylene glycol) fumarate) and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers, and nerve cells contained within or attracted to the matrix. The oligo(poly(ethylene glycol) fumarate) may be provided as a hydrogel. The matrix may be prepared by photocrosslinking the oligo(poly(ethylene glycol) fumarate) and the charged reactant in the presence of a photoinitiator. The nerve cells may be encapsulated in the matrix, or the nerve cells may be adhered to a surface of the matrix. The matrix may be prepared by crosslinking the oligo(poly(ethylene glycol) fumarate) and the charged reactant in the presence of a porogen. The scaffold may have a porosity of 70% to 90%.

[0040] The invention also provides a method for nerve tissue regeneration. In the method, a material including (i) a copolymer formed by reacting a first reactant selected from the group consisting of monomers, oligomers and polymers and a second reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers, and (ii) nerve cells is provided. The material is injected into a location in a patient's body, and the material crosslinks in the patient's body. The material may include a photoinitiator, and the step of crosslinking may comprise photocrosslinking the material. The first reactant may be oligo(poly(ethylene glycol) fumarate) hydrogel, and the second charged reactant may be selected from unsaturated quaternary ammonium compounds.

[0041] The invention also provides another method for nerve tissue regeneration. In the method, a scaffold is prepared that includes (i) a biodegradable matrix including a copolymer formed by reacting a first reactant selected from the group consisting of monomers, oligomers and polymers and a second charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers, and (ii) nerve cells contained within or attracted to the matrix. The formed scaffold is then implanted into a patient's body. The first reactant may be an oligo(poly(ethylene glycol) fumarate) hydrogel, and the charged reactant may be selected from unsaturated quaternary ammonium compounds. In one form, the scaffold is a nerve conduit.

[0042] Non-limiting example photoinitiators include 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one, acetophenone, benzophenone, and the benzoin ethers. Preferably, the photoinitiators are cytocompatible. N-vinyl pyrrolidinone (NVP) can be used as an accelerator for photocrosslinking. Other non-limiting example accelerators include N,N dimethyl toluidine or tetramethylethylenediamine. Preferably, the material photocrosslinks in the temperature range of 30°C to 45°C such that photocrosslinking at human body temperatures is possible. In one example form, a weight ratio of oligo(poly(ethylene glycol) fumarate) to charged monomer in the material is in the range of 1:0.01 to 1:0.5.

[0043] In one example of the invention, oligo(poly(ethylene glycol) fumarate) is copolymerized with [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC) to produce a positively charged hydrogel. This hydrogel can be crosslinked with light and/or redox-initiated systems and fabricated as sheet, sponge, and microspheres. Photocrosslinking can provide advantages such as spatial and temporal control over conventional crosslinking. Initiation does not require elevated temperature and polymerization rate is sufficiently rapid under physiological condition for in vivo placement.

[0044] In one example of photocrosslinking, we employed a long wavelength UV source and Irgacure® 2959 radical photoinitiator which has been reported as cytocompatible for crosslinking of OPF. (See, Bryant *et al.*, "Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts in vitro", *J Biomater Sci Polym Ed* 2000;11(5):439-57.) N-vinyl pyrrolidinone (NVP) can be used as an accelerator for photocrosslinking. An accelerating role has been previously reported for NVP in photoencapsulation of pancreatic islets. (See, Cruise *et al.*, "A sensitivity study of the key parameters in the interfacial photopolymerization of poly(ethylene glycol) diacrylate upon porcine islets", *Biotechnol Bioeng* 1998;57(6):655-65.)

[0045] The charged hydrogels of the invention can be used to improve the regeneration of nerve cells. Nerve cells obtained by biopsy or cell culture may be cultivated. As defined herein, nerve cells include, without limitation, neurons isolated from tissues such as nerve cells from the central nervous system including brain and spinal cord neurons, and neurons of the peripheral nervous system including sensory and motor neurons, as well as nerve cells obtained from

established cell lines and maintained in culture, and recombinant nerve cells. The charged hydrogels can improve nerve cell adhesion and improve extension of neurites including dendrites and axons. The charged hydrogels can be used to improve the regeneration of nerve cells in vivo and in vitro, by attaching or abutting the nerve cells to the charged hydrogels.

[0046] In in vitro applications, nerve cells may be cultured by placing the nerve cells on the charged hydrogels of the invention. Optionally, the charged hydrogels may be blended into or coated on a polymeric support such as a film. Serum, serum substitutes, growth factors (e.g., nerve growth factor), hormones, and/or drugs can be used to enhance proliferation and regeneration of nerve cells. Nerve explants also may be cultured in vitro for implantation in vivo. For example, nerve explants may be isolated from mammalian tissue and cultured on the charged hydrogels.

[0047] The charged hydrogels of the invention may be implanted in vivo into a patient in need of repair of damaged nervous system tissue. Scaffolds for nerve tissue engineering can be coated with, or made of, charged hydrogels of the invention to enhance regeneration of implanted nerve cells or nerve cells which migrate into, attach and proliferate within the implanted scaffolds. Compositions which further promote nervous tissue healing, such as proteins, antibodies, nerve growth factors, and hormones can be applied together with the charged hydrogels, and can be entrapped in or chemically bonded (e.g., covalently bonded) to the charged hydrogels.

Examples

[0048] The following Examples have been presented in order to further illustrate the invention and are not intended to limit the invention in any way.

[0049] Oligo-(polyethylene glycol) fumarate (OPF), which is a biocompatible and biodegradable macromer is copolymerized with [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC) to produce a positively charged hydrogel, as a substrate to enhance nerve cells attachment and differentiation. It has been demonstrated that the swelling and mechanical properties of these hydrogels vary in a dose dependent manner with the change in hydrogel formulations. Additionally, this work evaluates the effect of localized positive charge on neurite outgrowth in culture to demonstrate that the positively charged hydrogel can be

used for stimulating in vivo nerve regeneration. It has been demonstrated that that a fully synthetic approach eliminates the need for collagen or RGD peptides for cell attachment and neurite outgrowth. It has been demonstrated that incorporation of positive charge in hydrogel promotes neurite outgrowth of dissociated dorsal root ganglion (DRG) cells and DRG explants in an in vitro system. Moreover, myelin formation by Schwann cells is assessed on charged hydrogels in an in vitro system using rat DRG neurons and Schwann cells.

[0050] The term "molecular weight" in this specification refers to "weight average molecular weight" ($M_w = \sum_i N_i M_i^2 / \sum_i N_i M_i$). Although weight average molecular weight (M_w) can be determined in a variety of ways, with some differences in result depending upon the method employed, it is convenient to employ gel permeation chromatography. As used herein, the term "number average molecular weight" (M_n) refers to the total weight of all the molecules in a polymer sample divided by the total number of moles present ($M_n = \sum_i N_i M_i / \sum_i N_i$). Although number average molecular weight can be determined in a variety of ways, with some differences in result depending upon the method employed, it is convenient to employ gel permeation chromatography.

Example 1

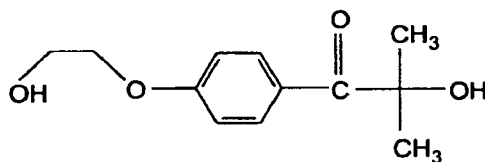
A. Macromer Synthesis

[0051] Oligo(poly(ethylene glycol) fumarate) (OPF) was synthesized using polyethylene glycol (PEG) (available from Aldrich) with the initial molecular weight of 10kDa according to a method published at Jo *et al.*, "Modification of oligo(poly(ethylene glycol) fumarate) macromer with a GRGD peptide for the preparation of functionalized polymer networks", *Biomacromolecules* 2001;2(1):255-61. See also Figure 1. The OPF had a weight average molecular weight (M_w) of 16246 ± 3710 .

B. Hydrogel Fabrication

[0052] Hydrogels were made by dissolving the OPF macromer with a final concentration of 33% (w/w) in deionized water containing 0.05% (w/w) Irgacure[®] 2959 radical photoinitiator (available from Ciba-Specialty Chemicals) and 0.33% (w/w) N-vinyl pyrrolidinone (NVP) as an accelerator for photocrosslinking. See Figure 2 which shows the reaction. The product sheet for Irgacure[®] 2959

describes Irgacure[®] 2959 as being 1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one and as having the following structure:



[0053] To produce positively charged OPF hydrogels, [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC) (75% available from Aldrich) at concentrations of 0.1, 0.2 and 1 M was added to the OPF solution. The OPF/MAETAC mixture was pipetted between glass slides with a 1 mm spacer and polymerized using 365 nm UV light at the intensity of $\sim 8 \text{ mW/cm}^2$ (Blak-Ray Model 100AP) for 30 minutes. ATR-FTIR data was generated and confirmed the presence of MAETAC on the crosslinked hydrogels.

C. Swelling Measurements

[0054] After crosslinking, the positively charged OPF hydrogels were cut into disks of 10 millimeters diameter with a cork borer and swollen in phosphate buffered saline (PBS) and deionized water for 24 hours at 37°C. Swollen samples were blotted dry and weighed (W_s), and then dried in reduced pressure and weighed again (W_d). The swelling ratio of the positively charged OPF hydrogels was calculated using the following equation: $\text{Swelling ratio} = (W_s - W_d) / W_d$. The swelling ratios of the positively charged OPF hydrogels increased in water significantly with the increase in concentration of MAETAC, while they remained constant in PBS, indicative of the ionic nature of the modified hydrogels. See Figure 3.

D. Compressive Modulus

[0055] Compressive modulus of the various swollen positively charged OPF hydrogels was determined using a dynamic mechanical analyzer (DMA-2980, TA Instruments) at a rate of 4 N/min. The modulus was determined as the slope of the stress versus strain curve at low strains (<20%). See Figure 4.

E. Cell Adhesion On Charged OPF Hydrogels

[0056] Figures 5A, 5B and 5C show that charge modification improves nerve cell attachment on the positively charged OPF hydrogels.

F. Cell Cultures and Neurite Growth

[0057] In order to determine the suitability of the positively charged OPF hydrogels for cell delivery, two types of dorsal root ganglion cell cultures were used. In the first cell culture, dissociated rat embryonic dorsal root ganglion neurons were grown on the positively charged OPF hydrogel for 7 days in the presence of nerve growth factor, and photographs were then taken. In the second cell culture, dorsal root ganglion explants were grown on the positively charged OPF hydrogel for a 24-40 hour period in the presence of nerve growth factor, and photographs were then taken. See Figures 6 and 7. Figure 8 shows dorsal root ganglion explants and neurite outgrowth on the positively charged hydrogels after 40 hours for a hydrogel without charge and for a hydrogel with 20% charge. The right photograph in Figure 8 shows significant neurite outgrowth.

[0058] Figure 9 shows dorsal root ganglion explant neurite outgrowth lengths on the positively charged hydrogels after 24 and 40 hours for a hydrogel without charge and for a hydrogel with 10% charge and for a hydrogel with 20% charge. The cells were cultured both with and without laminin. Figure 10 shows dorsal root ganglion explant neurite outgrowth lengths on the positively charged hydrogels after 40 hours for different molecular weight hydrogels. Figure 11 shows neurite length histograms for dorsal root ganglion explant grown on hydrogel without charge and for laminin derived peptide plastic and for a hydrogel with 10% charge and for a hydrogel with 20% charge.

[0059] Hydrogels with the incorporation of 10% and 20% charged monomer (MAETAC) show a significant increase in neurite outgrowth compared to the hydrogels without charge and laminin plastic as a positive control. A similar effect is seen on both hydrogels with lower and higher M_w . Neurite outgrowth observed on the charged hydrogels with and without laminin are similar. Laminin derived peptide improves nerve growth and is used in this experiment as a positive control. The neurite length histogram for dorsal root ganglion explant grown on 10% and 20% charged hydrogels is shifted to the higher neurite lengths compared to the histogram for dorsal root ganglion explant on hydrogels without charge and laminin derived peptide plastic as positive control.

[0060] Figure 12 shows the myelination of axons on charged hydrogel after 3 weeks. Dark stained nerve fibers showing myelination of the axons on charged hydrogel are seen in the pictures of Figure 12.

[0061] Figure 13 shows an OPF multi-lumen nerve guide fabricated with a stereolithography machine. It is a prototype of a 19 channel OPF-Hydrogel nerve conduit. The scaffold for nerve tissue regeneration may be a nerve conduit.

Example 2

A. Materials And Methods

i. Materials

[0062] OPF with molecular weight of $16,246 \pm 3,710$ was synthesized using PEG with molecular weight of 10 K according to a method previously described (see, Jo *et al.*, (2001) *Biomacromolecules* 2, 255-61). Briefly, 50 grams of PEG was azeotropically distilled in toluene to remove residual water and then dissolved in 500 milliliters distilled methylene chloride. The resulting PEG was placed in an ice bath and purged with nitrogen for 10 minutes, then 0.9 moles of triethylamine (TEA; available from Aldrich, Milwaukee, Wisconsin, USA) per mole of PEG and 1.8 moles of distilled fumaryl chloride (available from Acros, Pittsburgh, Pennsylvania, USA) per mole of PEG were added dropwise. The reaction vessel was then removed from the ice bath and stirred at room temperature for 48 hours. For purification, methylene chloride was removed by a rotary evaporator. The resulting OPF was dissolved in ethyl acetate and filtered to remove the salt from the reaction of TEA and chloride. The OPF was recrystallized in ethyl acetate and vacuum dried overnight.

ii. Gel Permeation Chromatography

[0063] The molecular weights of the OPF macromer and the PEG used for synthesis were measured with a Waters 717 Plus Autosampler gel permeation chromatography system (Milford, Massachusetts, USA) connected to a model 515 high-performance liquid chromatography pump and model 2410 refractor index detector. Monodispersed polystyrene standards (Polysciences, Warrington, Pennsylvania, USA) with number average molecular weights of 474, 6690, 18600, and 38000 g./mol. and polydispersities of less than 1.1 were used for the calibration curve. Three samples of each material were analyzed.

iii. Hydrogel Fabrication

[0064] Hydrogels were made by dissolving 1 gram of OPF macromer in deionized water containing 0.05% (w/w) of a photoinitiator (Irgacure® 2959, available from Ciba-Specialty Chemicals) and 0.3 gram of N-vinyl pyrrolidinone (NVP, available from Aldrich, Milwaukee, Wisconsin, USA as 1-vinyl-2-pyrrolidone). To produce positively charged hydrogels, [2-(methacryloyloxy)ethyl]-trimethylammonium chloride (MAETAC) (75%, available from Aldrich, Milwaukee, Wisconsin, USA) at different concentrations was added to the solution (as in Table 1). The OPF/MAETAC mixture was pipetted between glass slides with a 1 millimeter spacer and polymerized using UV light (365 nm) at an intensity of $\sim 8\text{mW/cm}^2$ (Black-Ray Model 100AP) for 30 minutes.

TABLE 1. Hydrogel Characteristics

Hydrogel	MAETAC Concentration (mM)	Equilibrium Swelling Ratio		Sol Fraction (%)	Compressive Modulus (kPa)
		Water	PBS		
HG-0	0	6.2±0.2	6.8±0.3	15.1±0.9	225±20
HG-10	200	7.6±0.1	6.2±0.1	10.2±2.3	331±25
HG-20	400	8.7±0.2	6.2±0.1	8.0±3.0	310±23
HG-30	600	9.2±0.2	6.1±0.2	9.1±2.6	324±28
HG-40	800	9.6±0.2	5.9±0.2	7.3±0.6	352±25

iv. Compression Testing

[0065] After crosslinking, hydrogels were cut into disks of 10-mm diameter with a cork borer and were swollen in phosphate-buffered saline (PBS, pH 7.0) for 24 hours. Compressive modulus of the various swollen hydrogels was determined using a dynamic mechanical analyzer (DMA-2980, TA Instruments, and New Castle, DE) at a rate of 4 N/min. The modulus was determined as the slope of the stress versus strain curve at low strains (<20%).

v. Swelling Measurements

[0066] OPF hydrogel disks (10 mm) were vacuum dried after fabrication, weighed (W_i) and swollen in either PBS or deionized water to equilibrium swelling (24 hours) at 37°C. Swollen samples were blotted dry and weighed (W_s), then dried in reduced pressure and weighed again (W_d). The swelling ratio and sol fraction of the hydrogels were calculated using the following equations:

$$\text{Swelling ratio} = (W_s - W_d) / W_d$$

$$\text{Sol Fraction} = ((W_i - W_d) / W_d) \times 100$$

vi. Attenuated Total Reflectance Fourier Transforms Infrared Microscopy

[0067] The surface of OPF hydrogels with and without modification was characterized using micro ATR-FTIR spectroscopy (Nicolet 8700). FTIR was coupled to a Continuum microscope (Thermo Electron Corp., Madison, Wisconsin, USA). The microscope utilized an ATR slide on germanium crystal and spectra were collected at the resolution of 4 cm⁻¹ or 128 scans with a sampling area of 150× 150 μm. Multiple spectra were collected on each hydrogel surface. The change in hydrogels surface composition was quantified by measuring the peak ratios of the characteristic peaks.

vii. Neuronal Cultures

[0068] Dorsal root ganglions (DRGs) were excised from E15 Sprague-Dawley rat pups (Harlan) trypsinized and mechanically dissociated; the cell suspension thus obtained was plated onto the hydrogels with different charges in MEM, (minimum essential medium, GIBCO) supplemented with 15% calf serum for dissociated or 10% calf serum for DRG explant cultures, nerve growth factor (NGF, 8ng/ml), glucose (0.6% w/v), and L glutamine (1.4 μM; Sigma). For dissociated cultures the DRG were treated with 0.25% trypsin in Hanks balanced salts for 30 minutes at 37°C and disrupted through a restricted glass pipette. The DRGs were then plated on collagen coated plates. Contaminating non-neuronal supporting cells were eliminated by treatment with 4μM 5 Fluoro-2-deoxy-uridine (FUDR)/ 4μM Uridine (Sigma, St. Louis, MO) which was added to the media and incubated in a humidified incubator at 37°C, 5% CO₂ for 3 to 5 days.

viii. DRG Explants

[0069] DRGs were dissected from E15 rat embryos and plated onto the test OPF hydrogel disks. DRGs explants were cultured on each hydrogel disks (10-15 per disk) with different charge and in vitro analysis and quantification of neurite extension on charged modified hydrogels were performed after 24 and 40 hours using a digital image analysis system of a Zeiss Axiovert 35 with a Nikon CCD camera. Light microscope images of the DRGs in culture were captured and eight longest neurites were traced and their lengths measured using Image J software obtained from the National Institutes of Health.

ix. Schwann Cell Cultures

[0070] Schwann cells were prepared from the sciatic nerve from 2 to 5 day old Sprague-Dawley rat pups, according to a previously published method (see, Wood, P. M. (1976) *Brain Res* 115, 361-75). Briefly, the stripped nerves were digested for 45 minutes in 0.25% trypsin / 0.03% collagenase in Hanks buffer, and mechanically dissociated. The subsequent cells suspension was plated on laminin coated Petri dishes in Dulbecco's Modified Eagles Medium/F12 (DMEM/F12, Gibco) supplemented with 10% fetal calf serum (FCS Gibco). The Schwann cells were grown for 48 hours to approximately 80% confluency before being trypsinized in trypsin/EDTA and the cells counted with a hemacytometer.

x. Neuron-Schwann Cell Cultures

[0071] These dissociated DRG cultures were not treated with FUDR, and maintained in a slightly modified dissociated DRG media. In this media, the calf serum is omitted and B27 supplement (Invitrogen) added, with an additional 70 µg/ml ascorbic acid. The media was changed 3 times a week for three weeks, before the cells were fixed with 4% paraformaldehyde in PBS and the cultures stained with Sudan Black as previously described (see, Carenini, *et al.*, (1998) *Glia* 22, 213-20).

xi. Statistical Analysis

[0072] All data for hydrogels characterization are reported as means ± standard deviations (SD) for n = 3 unless otherwise described differently in the experimental section. Single factor analysis of variance (ANOVA) was performed with StatView version 5.0.1.0 (SAS Institute, Inc, Cary, NC) to assess the statistical significance of the results. Bonferroni's method was employed for multiple comparison tests at significance levels of at least 95%.

B. Results

i. Hydrogel Characterization

[0073] OPF hydrogel was chemically modified with incorporation of a positively charged monomer MAETAC, which is a bifunctional molecule containing both a pH-independent cationic head (quaternary ammonium) and a reactive methacryl group that copolymerizes with the fumarate group of the OPF.

[0074] Fig. 14a shows the ATR-FTIR spectrum of unmodified OPF hydrogel after lyophilizing. Bands at 1650 and 1086 cm⁻¹ are assigned to carbonyl and C-

O-C of OPF, respectively. After copolymerization of the hydrogel with MAETAC, a new peak emerged at 1725cm^{-1} that is characteristic of methacroyl carbonyl from MAETAC (Fig. 14a). Peak heights of methacroyl group increased with increases in MAETAC concentration in the hydrogels formulation (Fig. 14b and 14c). The relative amount of MAETAC copolymerization in OPF hydrogels was calculated by comparing the ratios of the OPF absorbance (at the characteristic peak, near 1650 cm^{-1}) with the MAETAC absorbance (at the characteristic peak, near 1725 cm^{-1}) in the four hydrogels formulations (Fig. 14d). According to this analysis, the amount of MAETAC actually copolymerized within OPF hydrogels during the crosslinking process increased linearly ($R^2=0.998$) as the amount of MAETAC added to the formulation was increased. After hydration in either deionized water or PBS, most of the bands were broadened or disappeared. Three major bands, however, are seen at 3320 , 1650 and 1086 cm^{-1} assigned to hydroxyl, carbonyl and C-O, respectively (Fig. 15a). The peak heights at 1085 cm^{-1} assigned to C-O-C of PEG decreased with increase in MAETAC concentration in hydrogels suggesting that the chemical composition of the hydrogel surfaces changed due to the addition of MAETAC. The polar groups appeared to twist inward into the hydrogels bulk. Fig. 15b compares IR spectra of HG-20 hydrogel after hydration in deionized water and PBS. The C-O-C peak height at 1085 cm^{-1} in PBS appeared to be greater than that in deionized water, indicating that rotation of C-O-C in PBS is more restricted due to the presence of ionic moieties in MAETAC hydrogels. The peak ratios of hydrogels of all formulations at 1086 and 1650 cm^{-1} in PBS and deionized water are compared in Fig. 15c. After hydration in deionized water, $1085/1650$ peak ratio decreased with increasing MAETAC concentration, however decrease in peak ratio for the same samples in PBS was considerably lower. The swelling ratio, sol fraction and modulus of the hydrogels with four different concentrations of MAETAC are compared in Table 1. The sol fraction of the hydrogels after swelling in water decreased from $15.1\pm0.9\%$ to $7.3\pm0.6\%$ with addition of MAETAC to the hydrogels. It appears hydrogels crosslinking density increased with increasing MAETAC concentrations. In correlation with this finding, the compressive modulus of the hydrogel formulations significantly increased with addition of MAETAC. Unmodified OPF hydrogel had modulus of $225\pm20\text{ kPa}$; however, it increased to $331\pm25\text{ kPa}$ with the addition of

MAETAC (200 mM). Further addition of MAETAC did not affect compressive modulus of the hydrogels. The swelling ratios of the hydrogel formulations were measured in both deionized water and PBS. The swelling ratio of hydrogels increased slightly in deionized water as the concentration of MAETAC increased. For example, swelling ratio for HG-0 without MAETAC was (6.2 ± 0.2) which is significantly lower than that for all MAETAC hydrogels formulations ranging from 7.6-9.6. In PBS, unmodified OPF hydrogel had swelling ratio of (6.8 ± 0.3), and it did not change significantly with the addition of MAETAC to the formulations.

ii. Dissociated DRG Cells Attachment and Neurite Extension.

[0075] Dissociated DRG cells were seeded onto the hydrogels with different MAETAC concentrations. Photographs showed that cells attached to a greater extent on HG-10 and HG-20 hydrogels, while only few cells were observed on unmodified hydrogel. DRG cells extended their neurites more readily on the surface of positively charged hydrogels than unmodified hydrogel.

iii. DRG Explant Neurite Extension

[0076] In addition to the dissociated DRG cells, charged hydrogels also supported the growth of DRG explants dissected from rat embryos. In addition to neurons, these explants contained Schwann cells, fibroblasts, and other neuronal support cells. Photographs revealed that DRG explants attached to the charged surfaces and extended their neurites, while unmodified hydrogels did not support neurite extension from explants. Image analysis was used to quantify the neurite outgrowth on unmodified and charged modified hydrogels. The distributions of the neurite lengths for the different hydrogels formulations and laminin derived peptide coated plastic (LDP-plastic) were compared. These data show that the neurite distribution in the DRG explants on charged hydrogels was shifted to the right (longer neurites) compared with HG-0 without modification. It appears that neurite outgrowth on charged hydrogels HG-10 and HG-20 has distributed more uniformly and the median neurite lengths on charged hydrogels and LDP-plastic is significantly higher than those on HG-0. DRG neurite lengths on different hydrogel formulations and LDP coated plastic were compared. The results show that neurite lengths on HG-10, HG-20 and LDP coated plastic after 24 and 40 hours were significantly higher than that on unmodified hydrogel (HG-0) ($p < 0.05$).

Data also revealed that there was significant difference in neurite length between HG-20 and LDP coated plastic ($p < 0.05$).

iv. In Vitro Myelination

[0077] In addition to neurite extension, myelin formation by Schwann cells was investigated using rat DRG neurons and Schwann cells co-culture as described by Wood (see (1976) *Brain Res* 115, 361-75). After three weeks of culture, both Schwann cells and DRG neurons remained viable and DRGs extended neurites. Sudan black staining revealed that emerging neurites from DRGs were accompanied by migrating Schwann cells which were aligned along the neurites and differentiated into myelinating Schwann cells. Multiple myelinated internodes with nodes of Ranvier were seen in photographs.

C. Discussion

[0078] The aim of this study was to develop modified hydrogels with properties that can be varied in a controlled manner. Therefore, OPF macromer was copolymerized with a cationic monomer (MAETAC), which is pH independent and introduces a permanent charge into the OPF hydrogels in a dose-dependent manner. The effect of MAETAC incorporation was further investigated on surface and mechanical properties of the hydrogels. Neuronal cell attachment and neurite extension were analyzed as a specific response to the changes in the hydrogel charge densities. Moreover, in vitro myelination was investigated as a specific response of Schwann cell differentiation occurring on charged modified hydrogels.

[0079] In our study, primary sensory rat DRG neurons were used to analyze cell attachment and differentiation. DRG explants containing Schwann cells were also used to show the attachment of the primary nerve cells along with supporting cells that are critical for regeneration. Furthermore, neurite lengths were measured and compared between the hydrogels with different charge densities using DRG explants.

[0080] We demonstrated that MAETAC was successfully incorporated into the OPF hydrogel using ATR-FTIR, and the amount of MAETAC copolymerized within OPF hydrogel linearly increased with increasing MAETAC concentration in hydrogel precursor solution (Fig. 14). After hydration in deionized water, ATR-FTIR showed carbon oxygen (C-O-C) peak intensity decreased on hydrogel surfaces with increasing positively charged monomer concentration. However,

when hydrated in PBS, carbon oxygen (C-O) peak intensity on the MAETAC hydrogels increased (Fig. 15). The reorientation of the polar groups (C-O) to the hydrogel surfaces resulted in decreased swelling ratios of MAETAC hydrogels in PBS compared to those in deionized water. Furthermore, we demonstrated that incorporation of positively charged monomer promoted crosslinking of OPF hydrogels. With the addition of MAETAC monomer, sol fraction of OPF hydrogels significantly decreased indicating a highly crosslinked network (Table 1). This increase in crosslinking levels, led to the significant increase in compressive modulus of the OPF hydrogel. The swelling ratio of MAETAC hydrogels remained unchanged with increasing charged monomer concentration in hydrogel formulations (Table 1). It appears to be an equilibrium state for absorption of PBS due to the presence of ionic moieties in hydrogel backbone, where MAETAC hydrogels act as an active membrane for ion transport. This equilibrium state could be associated with the changes in osmotic pressure within the hydrogels network. These findings are in agreement with contact angle data that MAETAC hydrogels became more hydrophobic with the addition of MAETAC concentration in the hydrogels formulations when they were swollen in deionized water.

[0081] We demonstrated that charge modification of the OPF hydrogels improved neuronal cell attachment to the hydrogels in a dose dependent manner. Only few DRG cells were attached to unmodified OPF hydrogels while they attached to a greater extent to the charged hydrogel surfaces. A dense network of neurites emerging from DRG neurons was observed on HG-10 and HG-20 after 7 days in culture indicating differentiation of the neurons. In addition to primary sensory rat (DRG) neurons, our results revealed that charged hydrogels supported attachment of DRG explants containing Schwann cells. Incorporation of a positively charged monomer into the OPF hydrogel significantly enhanced attachment of DRG explants containing Schwann cells and neurite outgrowth. The neurite length for DRG explants grown on HG-20 ($723 \pm 54 \mu\text{m}$) was significantly higher than that on unmodified hydrogel ($0 \pm 0 \mu\text{m}$) and LDP coated plastic ($389 \pm 141 \mu\text{m}$) after 40 hours. These findings suggest that charged OPF hydrogels are capable of sustaining primary nerve cells and the support cells that are critical for regeneration. Previous studies have shown that polycationic polymers such as polylysine and polyornithine improved cell attachment and

neurite elongation in vitro, when used as coating materials. (See, Rauvala *et al.*, (1984) *J Cell Biol* 98, 1010-6; and Unsicker *et al.*, (1985) *Brain Res* 349, 304-8; and Letourneau *et al.*, (1975) *Dev Biol* 44, 92-101; and Crompton *et al.*, (2007) *Biomaterials* 28, 441-9). Other investigators have reported the repair of transected sciatic nerves in adult mice by electret guidance channels (see, Valentini *et al.*, (1989) *Brain Res* 480, 300-4). The nerves regenerated in positively charged channels contained significantly more myelinated axons than negatively charged or uncharged channels. Our findings in the present study also suggest a general relationship between positive electrical charges and enhancement of neural outgrowth. Although, the mechanism for bioeffects of electrical charge is not clear, it has been suggested that a permissive range of calcium ion is required for optimal neurite outgrowth. Since the influx of calcium ions across the cell membrane is regulated by the voltage-gated Ca^{2+} channels, interactions with an electrically charged substrate may lead to the changes in ionic flux. Thus, without intending to be bound by theory, MAETAC hydrogels may change calcium influx across the neurons membrane that leads to enhancement of neurite outgrowth. Changes in calcium concentrations may also influence the interaction with calmodulin, activate protein kinase C, or act directly on the activity of intercellular enzymes (see, Kater *et al.*, (1991) *J Neurosci* 11, 891-9). Other signal transductive candidates are membrane receptors whose interactions with G-proteins are modified by electrical stimulation or ligand-gated channels that are acted upon by the fields. Downstream to these membrane-located events in the nerve injury model are remodeling phases that may also be influenced by electric and electromagnetic fields, such as mitosis of Schwann cells, increased macrophage activity, upregulation of trophic factor(s) production, increased axonal transport, and basal lamina and cytoskeletal unit production.

[0082] In addition, charged hydrogels were analyzed for the appearance of myelinated structures in a co-culture containing DRG neurons and Schwann cells. In the peripheral nervous system myelin is formed and consists of highly specialized plasma membrane sheaths produced by differentiated Schwann cells. Synthesis, deposition, and organization of specific myelin components require an orchestrated series of cellular events to generate and maintain the ensheathment and compaction of myelin around the axon (see, Garbay *et al.*, (2000) *Prog*

Neurobiol 61, 267-304; and Givogri *et al.* (2000) *J Neurosci Res* 59, 153-9). In vitro myelination has been previously described on collagen I, in collagen gel or on PLL (see, Carenini *et al.*, (1998) *Glia* 22, 213-20; and Takeda *et al.* (2001) *Keio J Med* 50, 240-8). Here, we used Sudan black staining to assess DRGs formed myelin structures. We demonstrated that after 3 weeks in culture, both DRGs and Schwann cells remained viable on charged hydrogels (HG-10) and DRGs extended neurites. Sudan black staining revealed that emerging neurites from DRGs were accompanied by migrating Schwann cells which were aligned along the neurites and differentiated into myelinating Schwann cells. In addition, multiple myelinated internodes with nodes of Ranvier were seen over the myelinated axons.

[0083] In summary, the present study shows modification of photocrosslinkable OPF with positively charged monomer improved primary sensory rat (DRG) neurons attachment and differentiation in a dose dependent manner. Positively charged hydrogels also supported attachment of DRG explants containing Schwann cells and neuronal support cells that are critical for regeneration. Neurite extension was observed shortly after culturing the DRG explants on charged hydrogels and grew longer with time. Neurite lengths on charged hydrogels were significantly greater than on control groups. Moreover, charged hydrogels supported viability and differentiation of the neurons and Schwann cells in co-culture for a time period of three weeks. Myelinating axons were observed in the culture due the differentiation of Schwann cells to a myelinating phenotype. These findings suggest that charged OPF hydrogels are capable of sustaining primary nerve cells and the support cells that are critical for regeneration. This material is a suitable scaffold for in vivo nerve tissue engineering.

Example 3

A. Materials and Methods

i. Materials

[0084] OPF was synthesized from PEG molecular weight 10kDa and fumaryl chloride according to a published method (see, Jo S., *Macromolecules* 2001; 34:2839-2844).

ii. Hydrogel Fabrication

[0085] Hydrogels were crosslinked by dissolving OPF macromer to a final concentration of 33% (w/w) in deionized water containing 0.05% (w/w) of the photoinitiator Irgacure[®] 2959 (Ciba Specialty Chemicals) and 0.33% (w/w) of the co-monomer N-vinyl pyrrolidinone (NVP). In order to obtain positively charged hydrogels, [2-(methacryloyloxy) ethyl]-trimethyl ammonium chloride (MAETAC) was included in the formulation at the concentrations of 200 and 400 mM (HG-200 and HG-400, respectively). The hydrogel formulations were crosslinked using a 365 nm ultraviolet lamp with an intensity of $\sim 8\text{mW/cm}^2$ for 30 minutes.

iii. Swelling Ratio and Contact Angle

[0086] After crosslinking, the OPF hydrogels were cut into disks with the diameter of 10 millimeters and swollen in deionized water to equilibrium swelling (24 hours). Swollen samples were blotted, dried and weighed (W_s), and then vacuum dried and weighed again (W_d). Fold swelling of hydrogels was calculated using the following equation. ($n=3$):

$$\text{Fold swelling} = (W_s - W_d) / W_d$$

The water contact angle on the hydrogel disks was measured using sessile drop method with a goniometer (Krüss G10). All contact angles are mean values of five measurements on different parts of the hydrogel disk $\pm$ standard deviation.

iv. Tensile Testing

[0087] Once crosslinked, the samples were allowed to swell in PBS overnight to reach an equilibrium state. Dog-bone samples (ASTM D638-03, type IV specimens) were stamped out in the swollen state using a metal die. The samples were subjected to uniaxial tensile testing at a speed of 0.1 mm/s until fracture. The samples were misted with PBS throughout testing to maintain hydration. At least five samples were tested for each hydrogel formulation. The elastic modulus and ultimate tensile strength were determined from the stress-strain curves for each formulation.

v. DRG Explants

[0088] DRGs were dissected from E15 rat embryos and plated onto the test OPF hydrogel disks. DRG explants were cultured on each hydrogel disk with different charge, and in vitro analysis and quantification of neurite extension on

charged modified hydrogels were performed after 24 and 40 hours using a digital image analysis system of a Zeiss Axiovert 35 with a Nikon CCD camera.

B. Results and Discussion

[0089] Table 2 shows the swelling ratio of hydrogels increased in water significantly with the increase in concentration of MAETAC, while they remained constant in PBS, indicative of the ionic nature of the modified hydrogels. When swollen in water, water contact angle on OPF hydrogels increased with increasing MAETAC concentrations, although it remained unchanged when they were swollen in PBS. It appears hydrophobic groups migrate to the hydrogel surface due to the swelling in water. This result correlates well with swelling data that charged hydrogels swell more in water in comparison to PBS. Further investigations using ATR-FTIR confirmed reorientation of the functional groups on the hydrogel surfaces.

Table 2. Swelling Ratio And Contact Angle Of Charged Hydrogels

Hydrogel	Swelling Ratio		Contact Angle when Swollen in:	
	Water	PBS	Water	PBS
HG-0	6.2±0.2	5.9±0.8	32.0±2.5	44.0±2.0
HG-200	9.1±0.1	5.9±0.3	46.3±2.1	45.0±1.4
HG-400	10.4±0.1	5.7±0.1	57.7±2.7	46.3±1.5

[0090] Figure 16 shows Young's modulus of hydrogels with different formulations. As seen in this figure, photocrosslinkable OPF hydrogel had elastic modulus of 574 kPa and ultimate tensile stress of 284 kPa. Our data showed incorporation of positively charged monomer (MAETAC) did not have significant effect on ultimate tensile stress of the hydrogels with different formulation. However, elastic modulus of these hydrogels decreased significantly with the increase in concentration of MAETAC from 200 mM to 400 mM.

[0091] Phase contrast images of neurite outgrowth from DRG explants on a charged hydrogel were compared to the hydrogel without charge. These pictures showed that incorporation of positively charged monomer into the OPF hydrogel improved neurite outgrowth from DRG explant to a great extent.

[0092] Regardless of mechanical properties, neurite outgrowth was similar on both hydrogel formulations of HG-200 and HG-400, indicating that positive charge has a substantial role in nerve cell attachment and neurite outgrowth.

C. Conclusions

[0001] Our results showed modification of photocrosslinkable OPF with a positively charged monomer altered OPF hydrogels surface and bulk properties. These changes in hydrogel properties improved nerve cell attachment and neurite outgrowth. Thus, our findings support the use of charged hydrogels as a scaffold for nerve tissue engineering.

[0093] Therefore, the invention provides a biodegradable material for improving the regeneration of nerve cells. The material includes a copolymer formed by reacting a first reactant selected from monomers, oligomers and polymers and a second charged reactant selected from charged monomers, charged oligomers and charged polymers. Nerve cells are contained within or attracted to the copolymer. The first reactant may be oligo(poly(ethylene glycol) fumarate). The charged reactant may be selected from unsaturated quaternary ammonium compounds. The material may include a photoinitiator such that the material is photocrosslinkable. The material may include a bioactive agent such as a nerve growth factor. In one form, the material is a hydrogel that can be injected as a fluid into a patient's body via minimally invasive arthroscopic techniques to form a scaffold for nerve tissue regeneration.

INDUSTRIAL APPLICABILITY

[0094] The present invention relates to biodegradable hydrogels for improving the regeneration of nerve cells.

[0095] Although the present invention has been described in considerable detail with reference to certain embodiments, one skilled in the art will appreciate that the present invention can be practiced by other than the described embodiments, which have been presented for purposes of illustration and not of limitation. Therefore, the scope of the appended claims should not be limited to the description of the embodiments contained herein.

CLAIMS

What is claimed is:

1. A biodegradable material for improving the regeneration of nerve cells, the material comprising:
a copolymer formed by reacting (i) a first reactant selected from the group consisting of monomers, oligomers and polymers, and (ii) a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers; and
nerve cells contained within or attracted to the copolymer.
2. The material of claim 1 wherein:
the nerve cells are neurons.
3. The material of claim 1 wherein:
the charged reactant is cationic.
4. The material of claim 1 wherein:
the charged reactant is selected from the group consisting of quaternary ammonium compounds.
5. The material of claim 1 wherein:
the charged reactant is selected from the group consisting of unsaturated quaternary ammonium salts.
6. The material of claim 1 wherein:
the charged reactant is selected from the group consisting of quaternary ammonium halides.
7. The material of claim 1 wherein:
the charged reactant is selected from the group consisting of acrylate and methacrylate monomers.

8. The material of claim 1 wherein:
the first reactant is oligo(poly(ethylene glycol) fumarate).
9. The material of claim 1 further comprising:
a photoinitiator such that the material is photocrosslinkable.
10. The material of claim 1 wherein:
the material photocrosslinks in the temperature range of 30°C to 45°C.
11. The material of claim 1 wherein:
the material is injectable.
12. The material of claim 1 further comprising:
a bioactive agent.
13. The material of claim 12 wherein:
the bioactive agent is a nerve growth factor.
14. The material of claim 1 further comprising:
myelinating cells.
15. The material of claim 1 further comprising:
a porogen.
16. The material of claim 1 wherein:
the first reactant is oligo(poly(ethylene glycol) fumarate).
the charged reactant is selected from the group consisting of unsaturated quaternary ammonium compounds, and
a weight ratio of oligo(poly(ethylene glycol) fumarate) to the charged reactant in the material is in the range of 1:0.01 to 1:0.5.

17. A photocrosslinkable, biodegradable material comprising:
a copolymer formed by reacting oligo(poly(ethylene glycol) fumarate) and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers;
nerve cells; and
a photoinitiator.
18. The material of claim 17 wherein:
the nerve cells are neurons.
19. The material of claim 17 wherein:
the charged reactant is cationic.
20. The material of claim 17 wherein:
the charged reactant is selected from the group consisting of unsaturated quaternary ammonium compounds.
21. The material of claim 17 wherein:
the charged reactant is selected from the group consisting of acrylate and methacrylate monomers.
22. The material of claim 17 wherein:
the material photocrosslinks in the temperature range of 30°C to 45°C.
23. The material of claim 17 wherein:
the material is injectable.
24. The material of claim 17 further comprising:
a bioactive agent.
25. The material of claim 24 wherein:
the bioactive agent is a nerve growth factor.

26. The material of claim 17 further comprising:
myelinating cells.
27. The material of claim 17 further comprising:
a porogen.
28. The material of claim 17 wherein:
a weight ratio of oligo(poly(ethylene glycol) fumarate) to the charged
reactant in the material is in the range of 1:0.01 to 1:0.5.

29. A biodegradable material comprising:
a hydrogel prepared by reacting oligo(poly(ethylene glycol) fumarate) and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers; and
nerve cells contained within or attracted to the hydrogel.
30. The material of claim 29 wherein:
the hydrogel comprises 95 weight percent or more water.
31. The material of claim 29 wherein:
the nerve cells are neurons.
32. The material of claim 29 wherein:
the hydrogel includes a bioactive agent.
33. The material of claim 32 wherein:
the bioactive agent is a nerve growth factor.
34. The material of claim 29 wherein:
the hydrogel is photocrosslinkable in an aqueous solution.
35. The material of claim 29 wherein:
the charged reactant is selected from the group consisting of unsaturated quaternary ammonium compounds.
36. The material of claim 29 wherein:
the charged reactant is selected from the group consisting of acrylate and methacrylate monomers.
37. The material of claim 29 further comprising:
myelinating cells.

38. A scaffold for nerve tissue regeneration, the scaffold comprising:
a biodegradable matrix prepared by reacting oligo(poly(ethylene glycol) fumarate) and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers; and
nerve cells contained within or attracted to the matrix.

39. The scaffold of claim 38 wherein:
the oligo(poly(ethylene glycol) fumarate) is provided as a hydrogel.

40. The scaffold of claim 38 wherein:
the matrix is prepared by photocrosslinking the oligo(poly(ethylene glycol) fumarate) and the charged reactant in the presence of a photoinitiator.

41. The scaffold of claim 38 wherein:
the nerve cells are encapsulated in the matrix.

42. The scaffold of claim 38 wherein:
the nerve cells are adhered to a surface of the matrix.

43. The scaffold of claim 38 wherein:
the matrix is prepared by crosslinking the oligo(poly(ethylene glycol) fumarate) and the charged reactant in the presence of a porogen.

44. The scaffold of claim 43 wherein:
the scaffold has a porosity of 70% to 90%.

45. The scaffold of claim 38 wherein:
the scaffold is a nerve conduit.

46. The scaffold of claim 38 further comprising:
myelinating cells.

47. A method for nerve tissue regeneration, the method comprising:
providing a material including (i) a copolymer formed by reacting a first reactant selected from the group consisting of monomers, oligomers and polymers and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers, and (ii) nerve cells;
injecting the material into a location in a patient's body; and
crosslinking the material.

48. The method of claim 47 wherein:
the material further includes a photoinitiator, and
the step of crosslinking comprises photocrosslinking the material.

49. The method of claim 47 wherein:
the first reactant is oligo(poly(ethylene glycol) fumarate).

50. The method of claim 49 wherein:
the oligo(poly(ethylene glycol) fumarate) is provided as a hydrogel.

51. The method of claim 45 wherein:
the charged reactant is selected from the group consisting of unsaturated quaternary ammonium compounds.

52. The method of claim 47 wherein:
the nerve cells are neurons.

53. The method of claim 47 wherein:
the material further includes myelinating cells.

54. A method for nerve tissue regeneration, the method comprising:
providing a scaffold comprising (i) a biodegradable matrix including a copolymer formed by reacting a first reactant selected from the group consisting of monomers, oligomers and polymers and a charged reactant selected from the group consisting of charged monomers, charged oligomers and charged polymers, and (ii) nerve cells contained within or attracted to the matrix; and
implanting the scaffold into a patient's body.

55. The method of claim 54 wherein:
the first reactant is oligo(poly(ethylene glycol) fumarate).

56. The method of claim 55 wherein:
the oligo(poly(ethylene glycol) fumarate) is provided as a hydrogel.

57. The method of claim 54 wherein:
the charged reactant is selected from the group consisting of unsaturated quaternary ammonium compounds.

58. The method of claim 54 wherein:
the nerve cells are neurons.

59. The method of claim 54 wherein:
the material further includes myelinating cells.

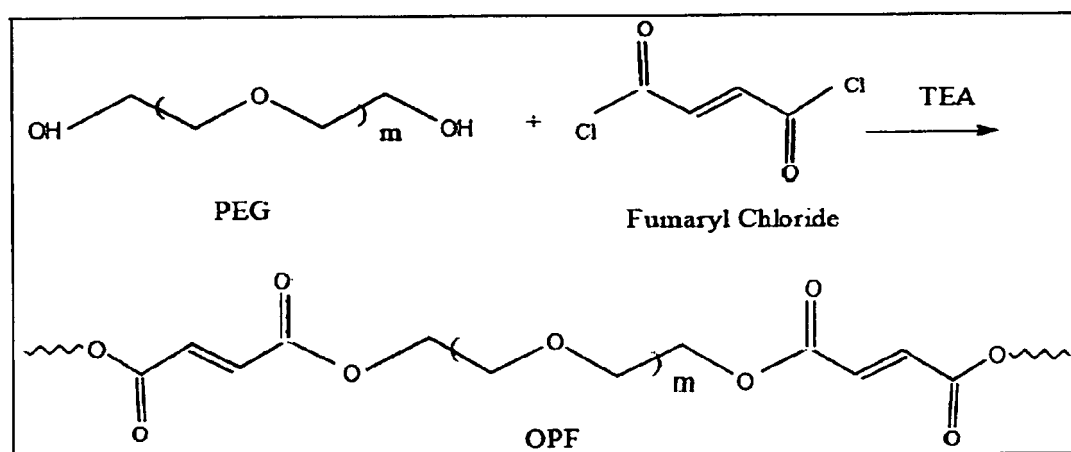


Figure 1

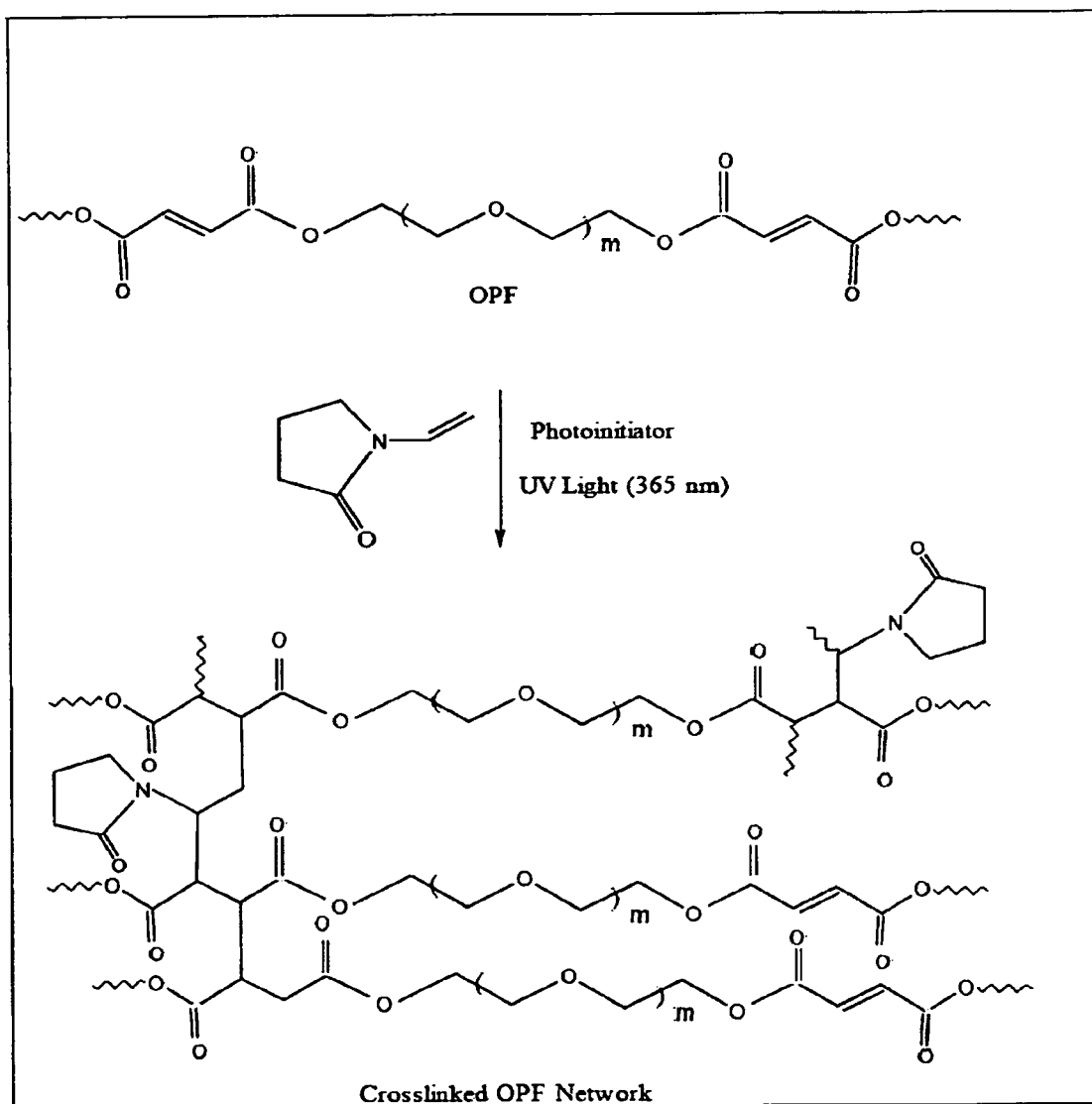
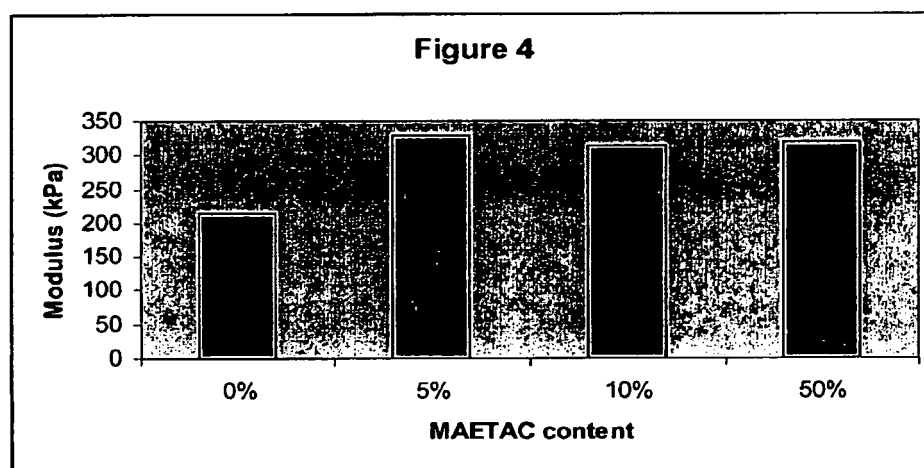
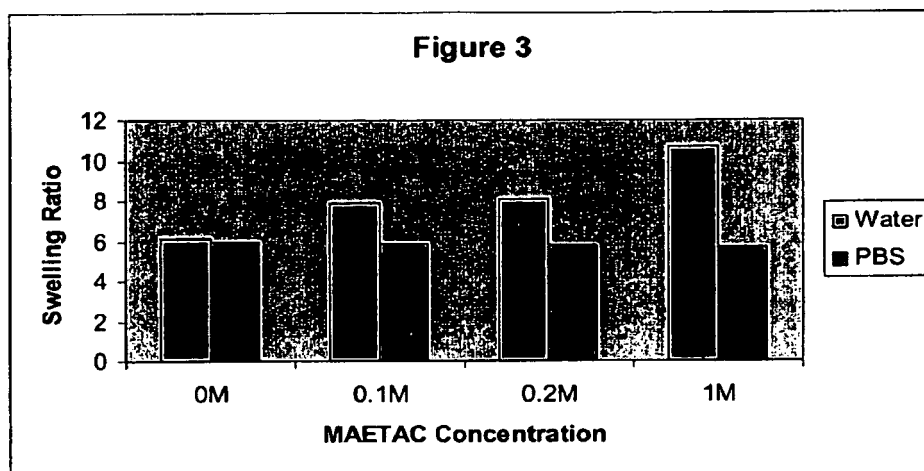
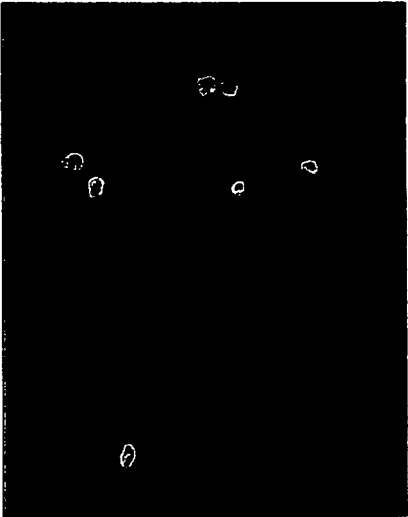


Figure 2

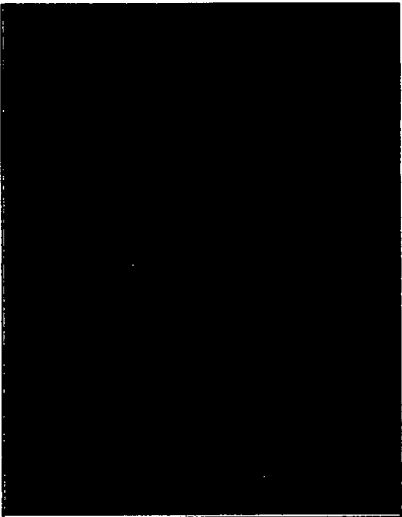


Charge Modification Improves Nerve Cell Attachment on Hydrogel



PC12 Cells on Hydrogel
without Charge

Figure 5A



PC12 Cells on Charged
Modified Hydrogel

Figure 5B



GFP Schwann Cells on
Charged Modified Hydrogel

Figure 5C

Figure 6

Dorsal Root Ganglion (DRG) Cultures:



E15 rat embryo



Spinal cord with 2 DRG

Two types of DRG cell cultures were used:

1-Dissociated DRG in the presence of nerve growth factor (NGF) over a 7 days period

2-DRG explant for measuring neurite outgrowth in the presence of NGF over a 24 -40 hour period

Figure 7
Dissociated DRG Cell Attachment and Neurite Outgrowth
on Charged Modified Hydrogel after 7 Days

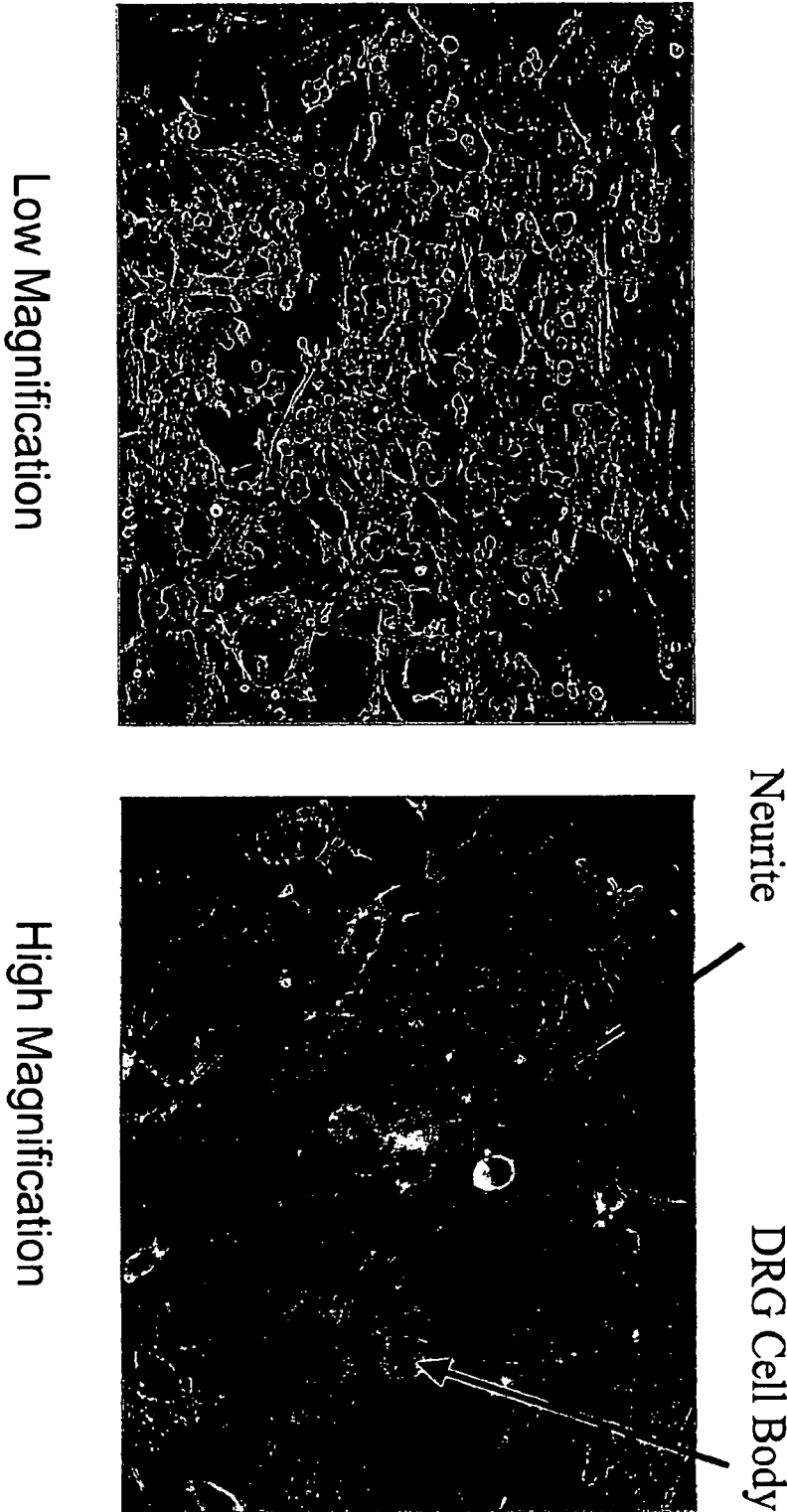


Figure 8

DRG Explant and Neurite Outgrowth on Positively Charged Hydrogels after 40 hours

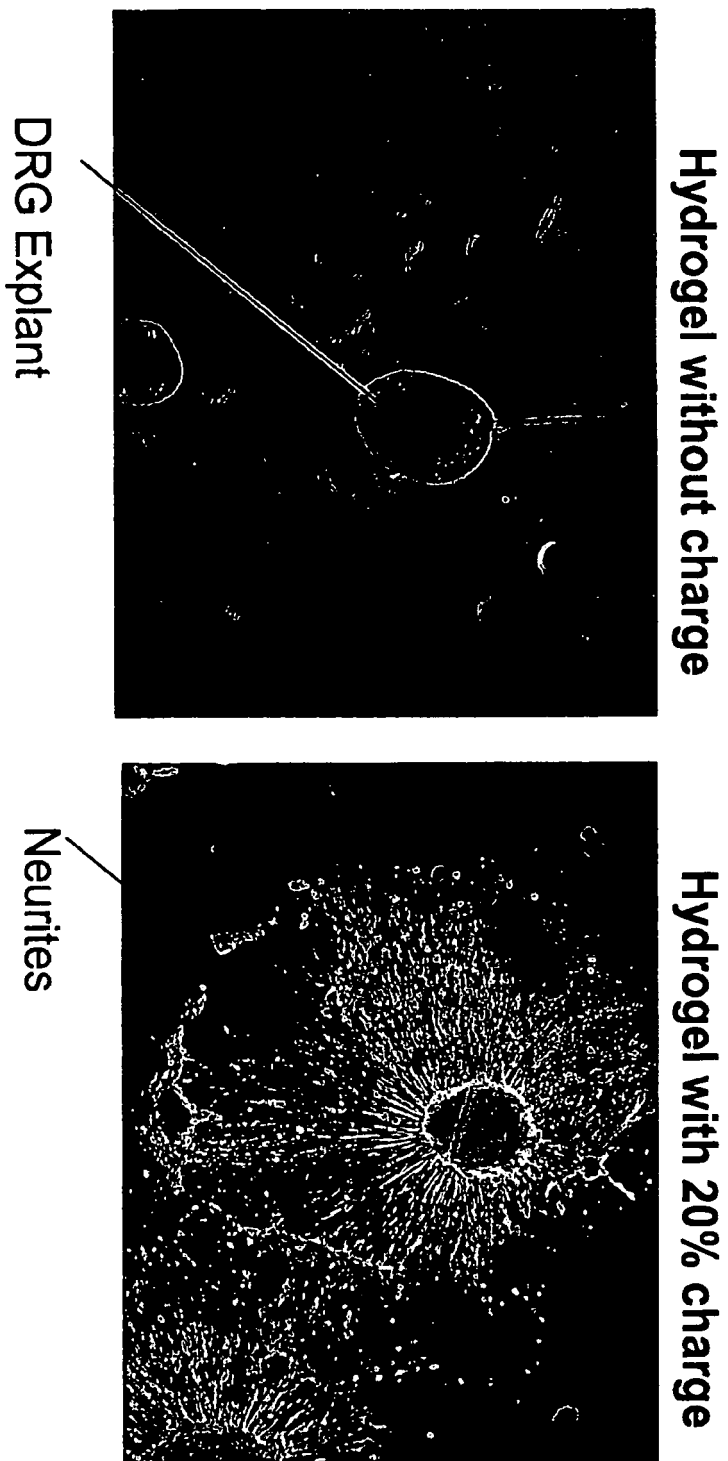


Figure 9
DRG Explant Neurite lengths on Positively Charged
Hydrogels after 24 and 40 hours

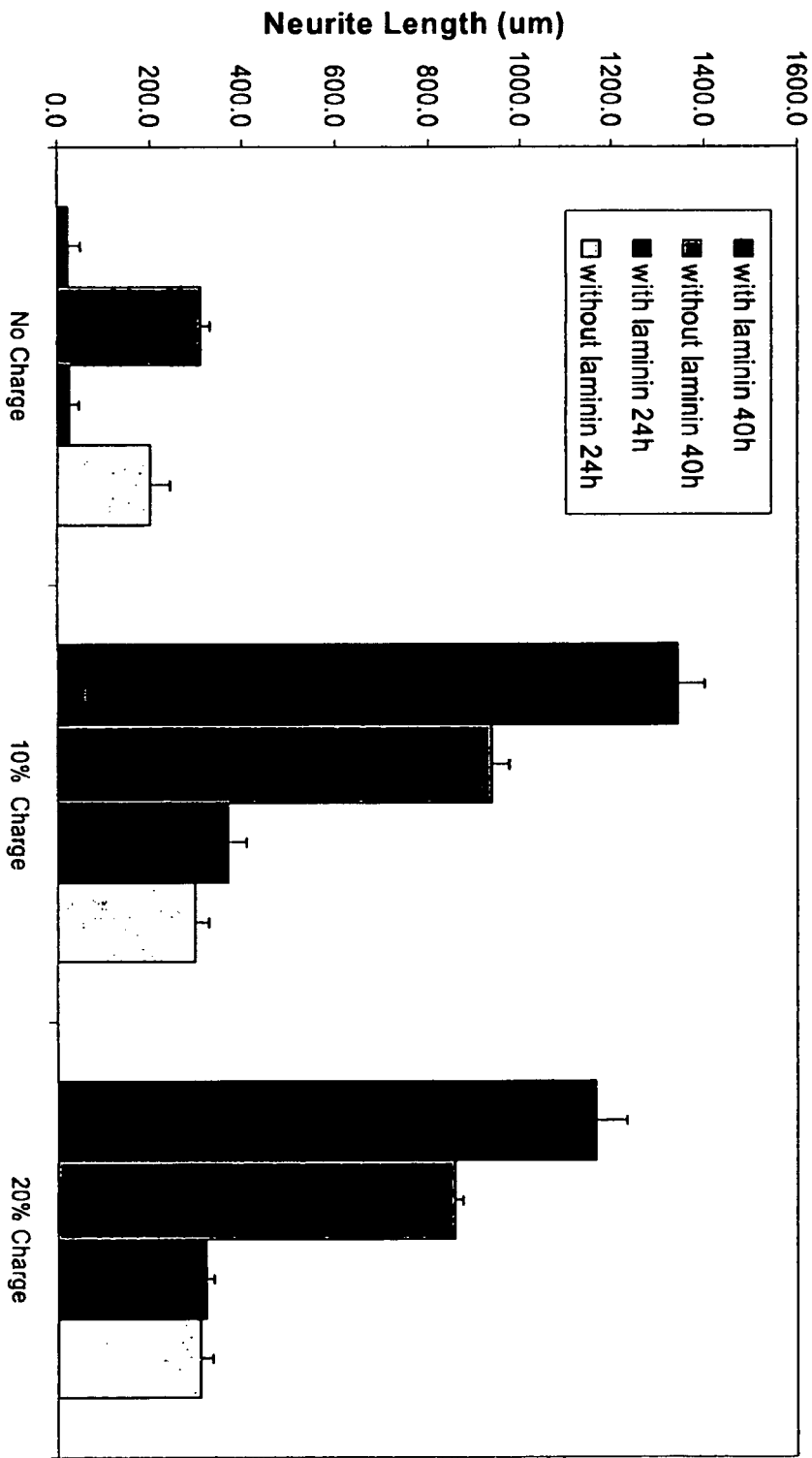
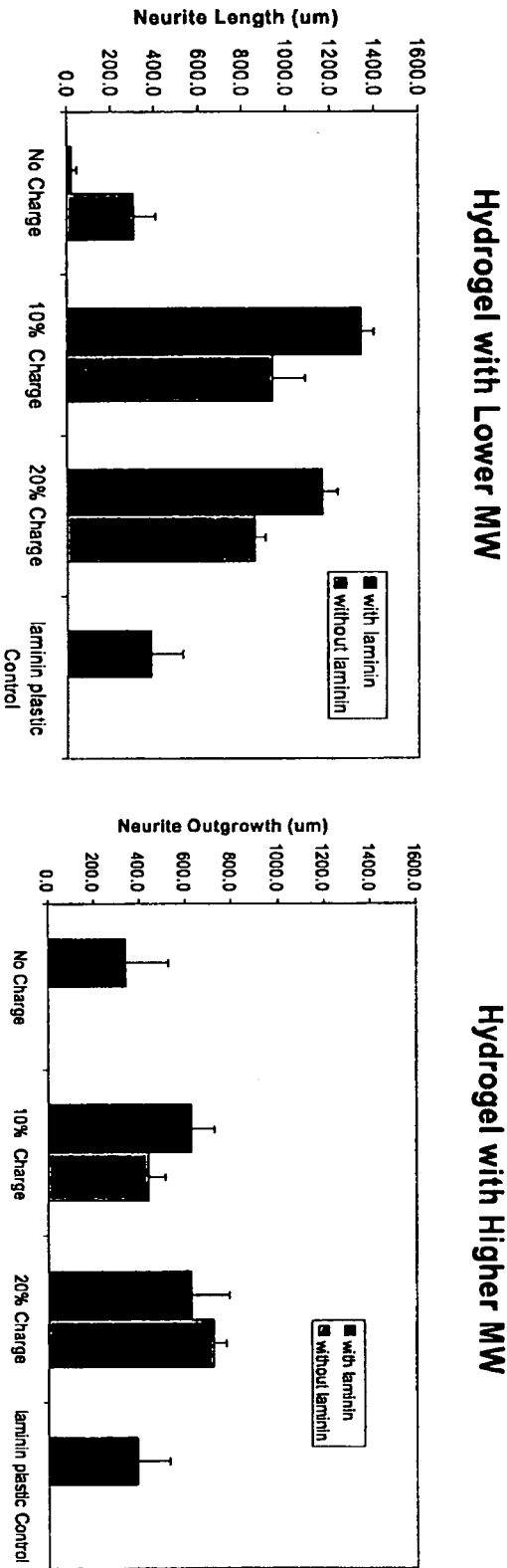
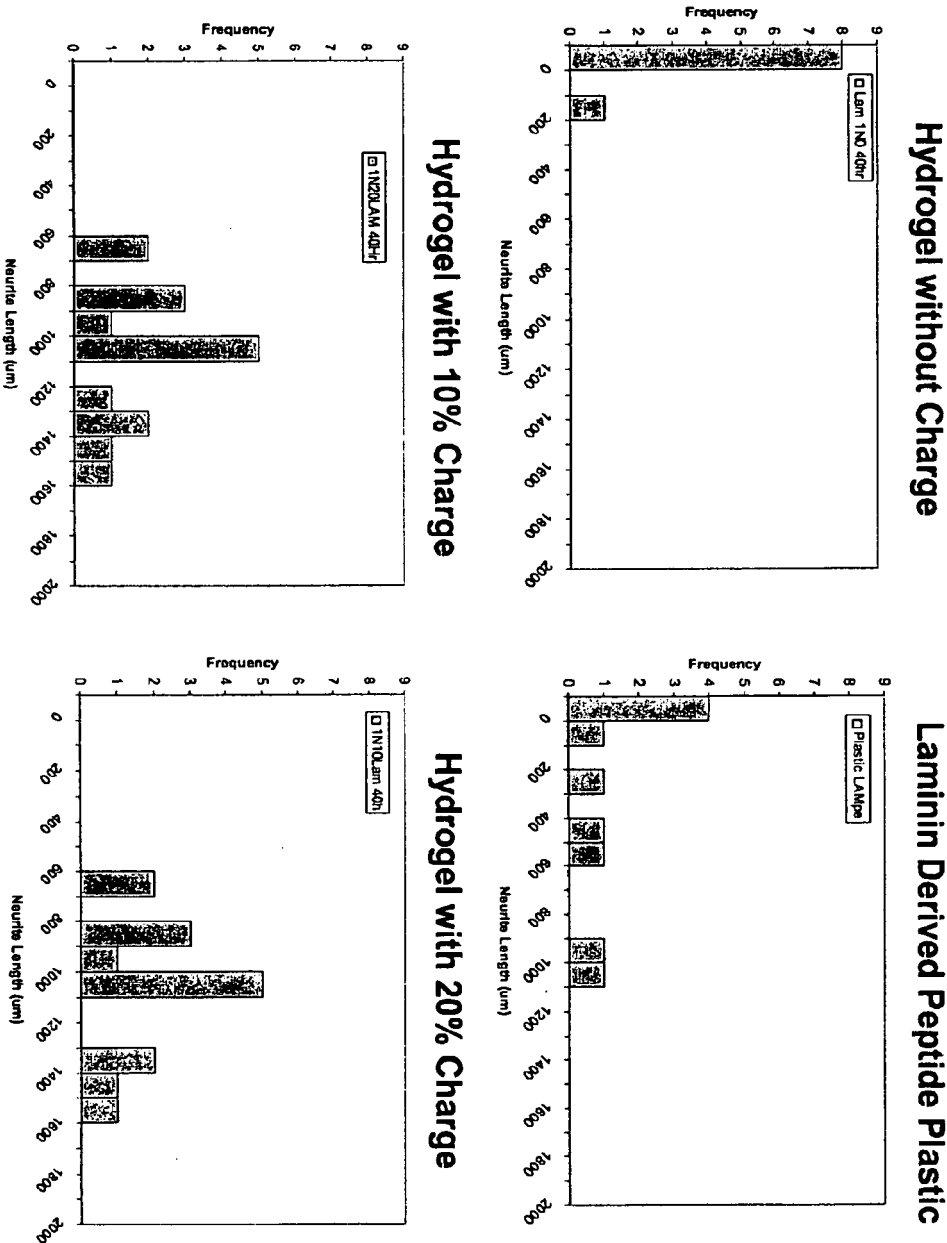


Figure 10 - DRG Explant Neurite lengths on Positively Charged Hydrogels after 40 Hours



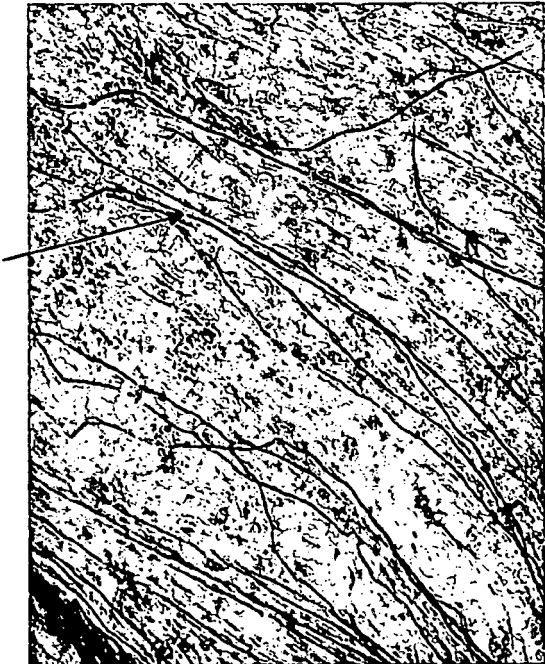
- Hydrogels with incorporation of 10% and 20% charged monomer (MAETAC) shows significant increase in neurite outgrowth compared to the hydrogels without charge and laminin plastic as positive control.
- Similar effect is seen on both hydrogels with lower and higher MW.
- Neurite outgrowth observed on the charged hydrogels with and without laminin are similar.
- Laminin derived peptide improves nerve growth and is used in this experiment as positive control.

**Figure 11 - Frequency of Neurite Outgrowth for DRG
Explant Grown on Charged Hydrogels**



The neurite length histogram for DRG explant grown on 10% and 20% charged hydrogels is shifted to the higher neurite lengths compared to histogram for DRG explant on hydrogels without charge and laminin derived peptide plastic as positive control

Figure 12 - Myelination of Axons on Charged Hydrogel after 3 Weeks



Myelinated Axons

Low Magnification



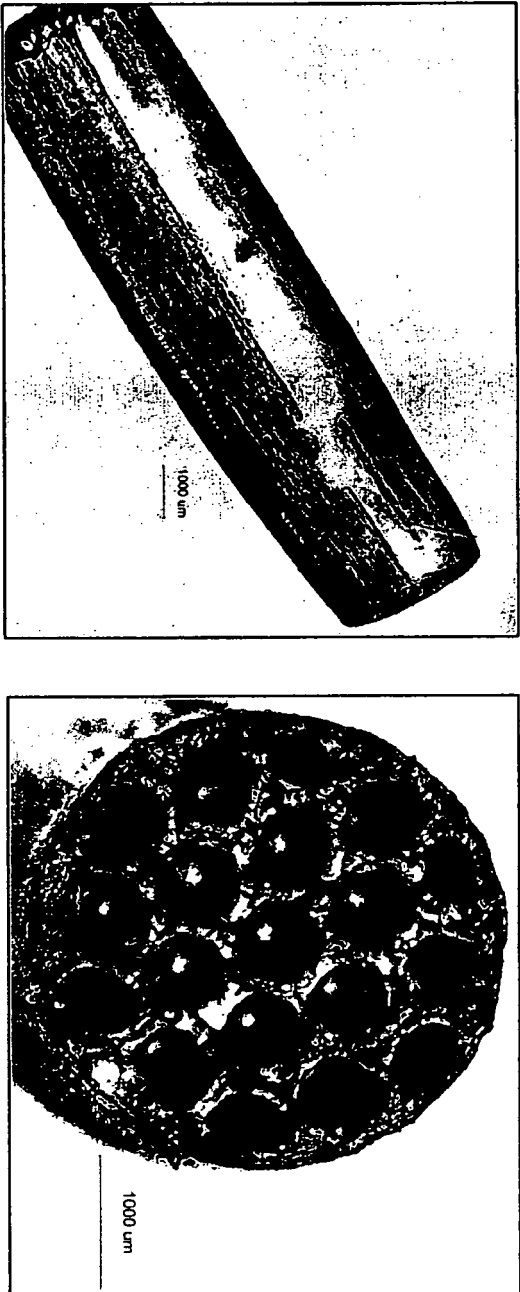
Myelinated Axons

Schwann Cells

High Magnification

Dark stained nerve fibers showing myelination of the axons on charged hydrogel are seen in the pictures

**Figure 13 - OPF Multi-lumen Nerve Guide Fabricated
with Streolithography Machine**



Prototype of a 19 channel OPF-Hydrogel Nerve Conduit

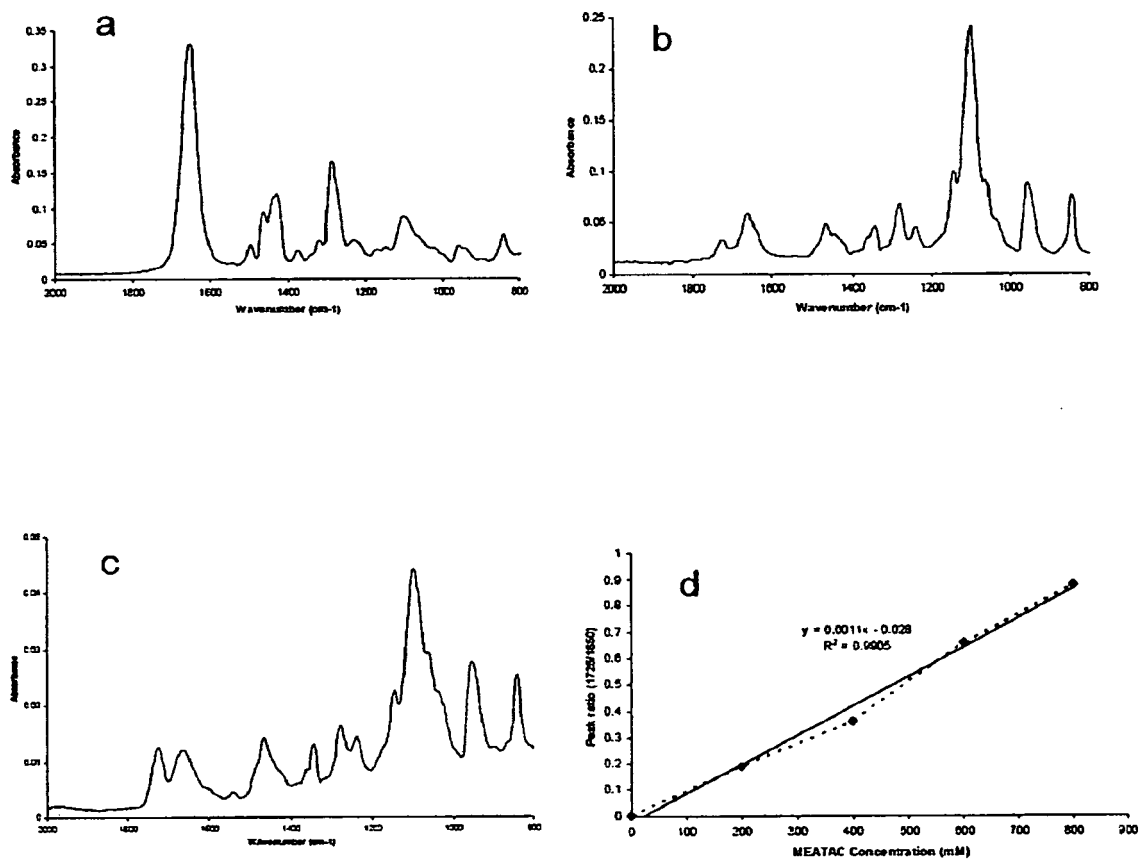


Figure 14

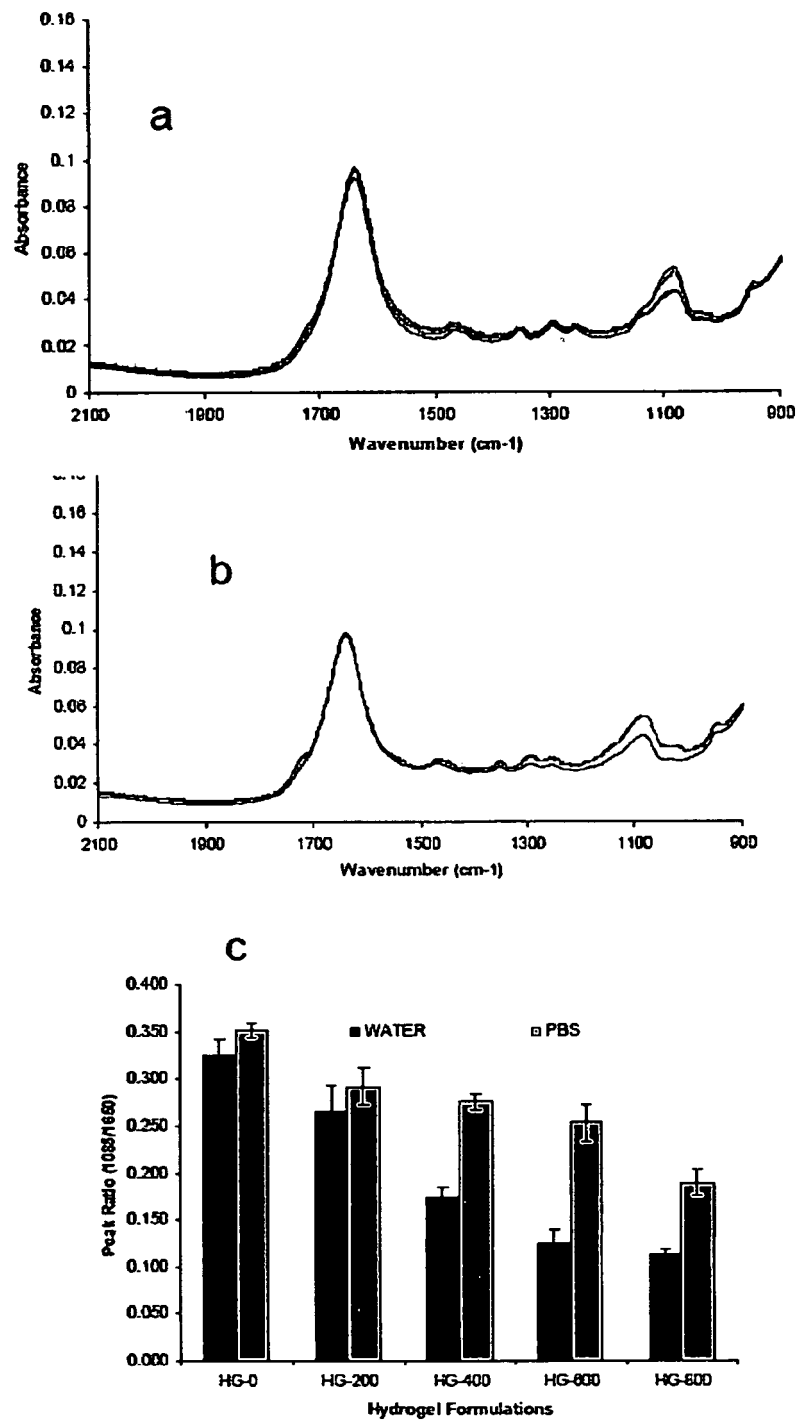


FIGURE 15

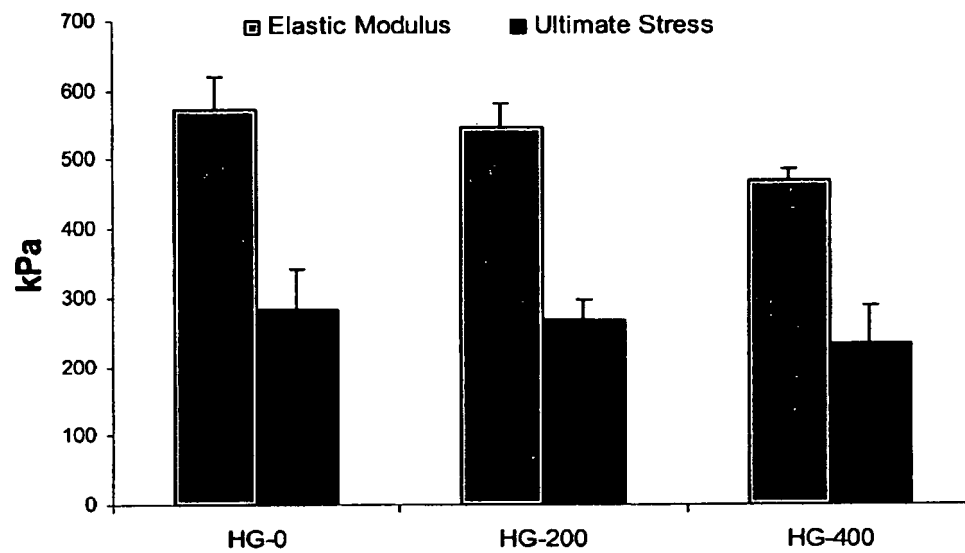


FIGURE 16