An injectable neurotrophic factor delivery system supports retinal ganglion cell survival and regeneration following optic nerve crush

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Materials and Methods

Materials

N-BOC-Serinol, urea, hexamethylene diisocyanate (HDI), anhydrous chloroform, and anhydrous N,N-dimethylformamide (DMF), N-isopropylacrylamide (NIPAAm), paraformaldehyde (PFA), sucrose, bovine serum albumin (BSA), and 4,4‘-azobis(4-cyanovaleric acid) (ACA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(3-Dimethylaminopropyl)-N- ethylcarbodiimide hydrochloride (EDC), potassium tert-butoxide (t-BuOK), 1,3-propane sultone (PS), N-hydroxysuccinimide (NHS), and trifluoroacetic acid (TFA) were purchased from Alfa Aesar (Ward Hill, MA, USA). Anhydrous diethyl ether was purchased from Fisher Scientific (Pittsburgh, PA, USA). Anhydrous dichloromethane (DCM) was purchased from JT Baker (Phillipsburg, NJ, USA). Dialysis tube (Spectra/ Por) was obtained from Spectrum Labs (Houston, TX). Phosphate Buffered Saline (PBS) was purchased from Thermo Scientific. IRDye 800CW NHS Ester was purchased from LI-COR (Lincoln, NE). Infrared (IR) Dye 800 CW NHS ester (LI-COR 929-70020) was purchased from LI-COR (Lincoln, NE, USA). Zeba Spin Desalting Columns (89882) were purchased from ThermoFisher Scientific (Waltham, MA, USA). Wistar rats were purchased from Charles River Laboratories (Wilmington, MA, USA). Sterile saline, isoflurane, ketoprofen, and xylazine were purchased from MWI Veterinary Supply (Boise, ID, USA). Optimal cutting temperature (OCT) compound was purchased from Sakura (Torrance, CA, USA). Superfrost glass slides were obtained from Fisher Scientific (Chicago, IL). GFAP (mouse IgG1), GAP-43 (Rabbit IgG), Alexa Fluor 488 (goat anti-mouse IgG), Alexa Fluor 594 (goat anti-rabbit IgG), and SlowFade Diamond antifade mountant with DAPI were purchased from Life Technologies (Carlsbad, CA, USA). Triton X-100 was purchased from MP Biomedicals.

Equipment

Lower critical solution temperature (LCST) measurements were made on a Cary 100 UV-visible spectrophotomer (Agilent Technologies, Inc., Santa Clara, CA). Elemental analysis of non-coated samples was performed by scanning electron microscope (SEM) (JEOL JSAM-6010la analytical scanning electron microscope, Peabody, MA, USA) in low-vacuum (20 kV) mode, and the analysis was carried out by energy dispersive X-ray spectrometry (EDX system by EDAX). In-vitro release of CNTF concentration was detected using fluorescence readings from the LI-COR Odyssey Classic (Lincoln, NE). 32 gauge needles used for the injection (TSK Laboratory (Tochigi-shi, Tochigi-ken, Japan). Tissue was sectioned using a CryoStar NX70 Cryostat. Confocal images were collected using a Nikon Eclipse Ti C2 LUN-A microscope (Nikon, Tokyo) equipped with two C2-DU3 high sensitivity PMT detectors, 4 diode lasers (405/488/561/640 nm), and a motorized microscope stage with 3-axis navigation (X, Y, and Z) controlled by the NIS-Elements software package. Laser and software setting were kept constant between specimens and to allow for comparison of different image acquisitions.

Elemental analysis

Polymer solutions were prepared in ultra pure water and allowed to gel at 37 °C for 15 min. The gelled samples were then frozen quickly using liquid nitrogen, cut in half to

expose the center structure, and rapidly transferred to a freeze-dryer for 24 h (-48 °C, 38 × 10^{-3}). An electron beam was scanned across a dried sample of both Sul-PSHU-PNIPAAm and PSHU-PNIPAAm. When the beam strikes the sample, signals are produced that are representative of a sample's elemental composition.

Solution to gel phase transition.

Lower critical solution temperature (LCST) was used to analyze the gelling properties of the thermally reversible injectable scaffold. 1% (wt/v) PSHU-PNIPAAm and PSHU-PNIPAAm-RGD were be loaded in a temperature-controlled UV/visible spectrophotometer. The transmittance through the polymer solution at 480 nm was monitored as the temperature increases from 15 °C to 45 °C. The rate of temperature increase was held at 2°C/min. This constant rate allowed us to observe the gelation time of both SRTG and RTG for a better comparison. Gelation activity was indicated by an increase in opaqueness and therefore a decrease in transmittance.

IR-Dye conjugation to CNTF.

CNTF was conjugated to IR Dye 800 CW NHS ester using a modified version of the IRDye 800 CW Microscale protocol provided by LI-COR. Briefly, lyophilized IR Dye was reconstituted in DMSO at a concentration of 15 mg/mL and then diluted to a working concentration of 0.15 mg/mL in PBS. Lyophilized CNTF was reconstituted in 1X PBS at a concentration of 0.1 mg/mL. The IR dye solution and CNTF solution were added to a microcentrifuge tube in a 1:50 volume ratio, respectively, at pH 8.5 and reacted for 2 hours with shaking, protected from light. After the reaction, excess IR dye was removed using Zeba Spin Desalting Columns to purify the IR-labeled CNTF.

In-vitro CNTF Release Test.

To a 1.5 mL centrifuge tube, 100 ng of IR-labeled CNTF was added to a solution of Sulfonated PSHU-PNIPAAm or PSHU-PNIPAAm for a final RTG concentration of 1% (mg/mL) with samples run in triplicate. This solution was allowed to dissolve overnight at 4°C protected from light. The following day, samples were gelled in an incubator (37°C, 5% CO_2) for 5 minutes to allow stable gel formation, followed by the addition of 600 µL of 1X PBS (37°C). At each time point, 300 µL of the release solution was gently mixed and removed from each sample and replenished with 300 µL of fresh PBS at 37°C. To a black, 96 well optical-bottom plate, 100 µL of each release sample was added. The fluorescence of each sample was measured using the LI-COR Odyssey Classic. Known concentrations of IR-labeled CNTF were added to the plate to generate a standard curve to calculate release sample concentrations.

Optic nerve crush model

To begin, rats were given intraperitoneal injections of a solution (1ml/kg) containing 1-2 mg/kg xylazine in sterile water. A lateral canthotomy was performed after anesthetizing the eye with proparicaine and then sterilizing the area surrounding the lateral corner of the eye with betadine. The eye was then irrigated using normal saline. Using a straight hemostat, the skin at the lateral corner of the eye was crimped all the way down to the orbit for 1 minute (to achieve hemostasis). The skin around the lateral orbit was lifted with forceps and a 0.5 cm incision was made with scissors. Blunt dissection was utilized

to expose the optic nerve. Using a number 5 jeweler's forceps, the optic nerve was crimped three times, 10 seconds each, approximately 2 mm behind the globe. Since a minimal incision was made, the procedure did not require sutures and healed properly on its own.

Retina and optic nerve immunohistochemistry (IHC).

At the appropriate time points (1 week, 2 weeks, or 4 weeks), rats were euthanized using an unchanged cage and a flow rate of CO_2 introduced to displace 20% of the cage volume per minute. A bilateral thoracotomy was performed as a secondary method of euthanasia. They eyes were then enucleated and fixed using 4 % PFA in PBS for 30 min, followed removal of the cornea and lens and an additional fixation period in 4% PFA for 30 min. The tissue was then cryoprotected with 10%, 20%, and 30 % sucrose in PBS for a total of 2 days, embedded in optimal cutting temperature (OCT) compound, and frozen at -80 °C. Longitudinal tissue sections for both analysis of the optic nerve (10 μm) as well as the retina (10 μm) were collected along the nasal-temporal plane using the optic cup as a reference point.
OCT was removed from the glass slides with a 5 min PBS wash. The slides were fixed in NBF for 10 min and washed 3 times in 0.01% tween in PBS for 3 minutes each. The sections were then permeabilized in 0.01% triton X-100 in PBS for ten minutes and washed 3 times in 0.01% tween in PBS 3 times for 3 minutes each. Non-specific binding was blocked by incubating sections in 2% BSA, 0.5% triton X-100, in PBS for 1 h at room temperature. Sections were then stained with the appropriate primary antibody GFAP (1:250), GAP-43 (1:250), or Brn3a (1:100) overnight at 4°C. All antibody dilutions were prepared in the blocking buffer. Slides were then washed with 0.01% tween in PBS 3 times for 3 minutes each and then incubated with the appropriate secondary (Alexa Fluor 488 (1:500) for GFAP, Alexa Fluror 546 (1:500) for GAP-43, and Alexa Fluror 594 (1:500) for Brn3a) prepared in the blocking buffer for 1 h at room temperature. Finally slides were and washed 3 times in 0.01% tween in PBS for 3 min each and then 3 times in dH2O for 3 min each. Stained slides were mounted with Fluoromount G with DAPI mounting medium. Slides were stored at -4°C.

Analyzing of neuronal survival

To determine the number of Brn3a positive cells following nerve crush and subsequent treatment, five images were taken from each area of each retina (two peripheral, two mid-peripheral, and optic nerve cup). As the number of RGCs may vary depending on the different areas of the retina, the region of the retina that was analyzed for Brn3a positive cells was held consistent by using the optic nerve cup as an anatomical marker. Only tissue sections taken from around the optic head were analyzed and used for Brn3a counting. Brn3a positive cell counts from all five images were performed using Fiji. The five cell counts from each retina were analyzed by averaging these values and comparing the cell count between each group (saline, free CNTF, PSHU-PNIPAAm-CNTF, Sul-PSHU-PNIPAAm-CNTF, and healthy eyes). Each data point represents the mean +/- SD of the surviving neurons post crush. Statistics were performed using a one-way ANOVA

–Tukey post hoc test, * p<0.01, ** p<0.001, ***p<0.0001 n=3-5 eyes/group/time-point.

Quantification of axonal growth

The extent of RGC axon growth was evaluated using GAP-43 immunostaining of longitudinal sections of the optic nerve (10 µm). Composite images of whole cross-sections of the retina and the optic nerve were imaged using 10x magnification (Nikon). Z-stack maximum intensity images of 5 total stacks were taken to encompass the full section height (10 µm). Axonal growth was quantified by determining the average level of fluorescence in regions of nerve 100 µm, 250 µm, 500 µm and 1000 µm downstream from the lesion site. During analysis, the injury site was determined in the same tissue section that had been stained with GAP-43 through dark field microscopy. Average pixel intensity for each retinal section was analyzed for n= 3-5 per group per time-point and expressed as mean ± S.E.M.

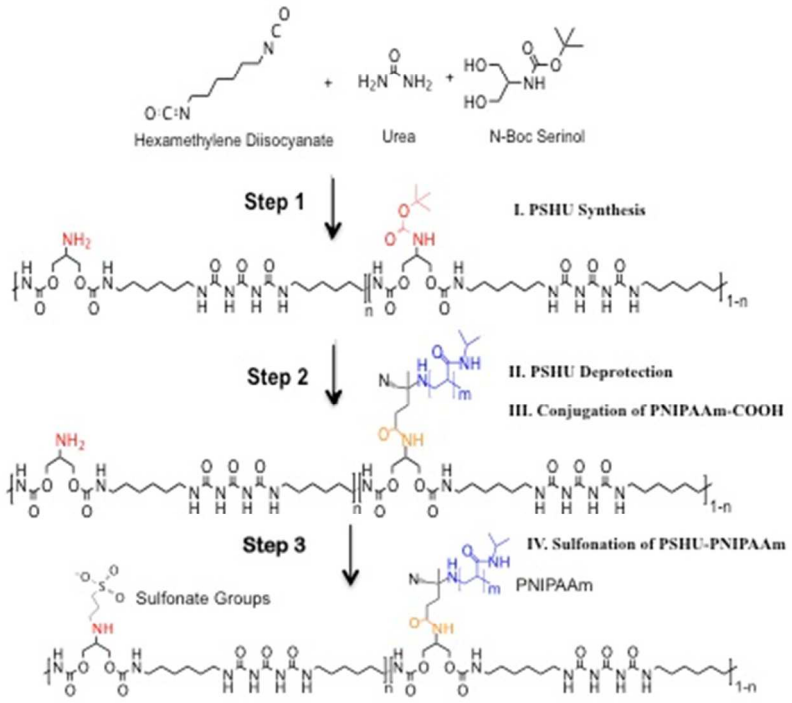
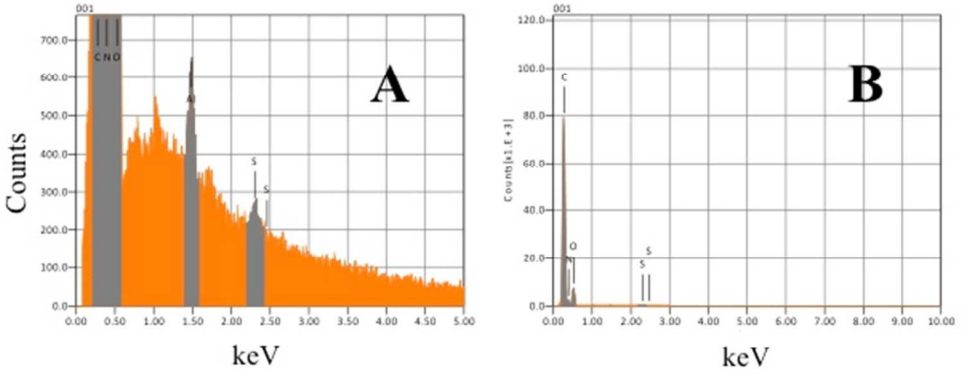


Fig S1. Schematic of synthesis process for Sul-PSHU-PNIPAAm (SRTG). Step 1) synthesis of the poly(serinol hexamethylene urea) PSHU; Step 2) conjugation of PNIPAAm-COOH; and Step 3) sulfonation of free amine groups.



SRTG			
Chemical Formula	Mass %	Atom %	Ratio
C	68.55	73.17	0.649
N	15.25	13.96	0.162
O	15.88	12.72	0.246
S	0.09	0.03	0.004

RTG			
Chemical Formula	Mass %	Atom %	Ratio
C	73.76	77.84	0.815
N	12.28	11.11	0.136
O	13.93	11.04	0.242
S	0.00	0.00	0.000

Fig S2. Elemental analysis of SRTG and RTG. SRTG showed a 0.09 mass% of sulfur present in the polymer compared to 0.00 mass% detected in the RTG polymer.

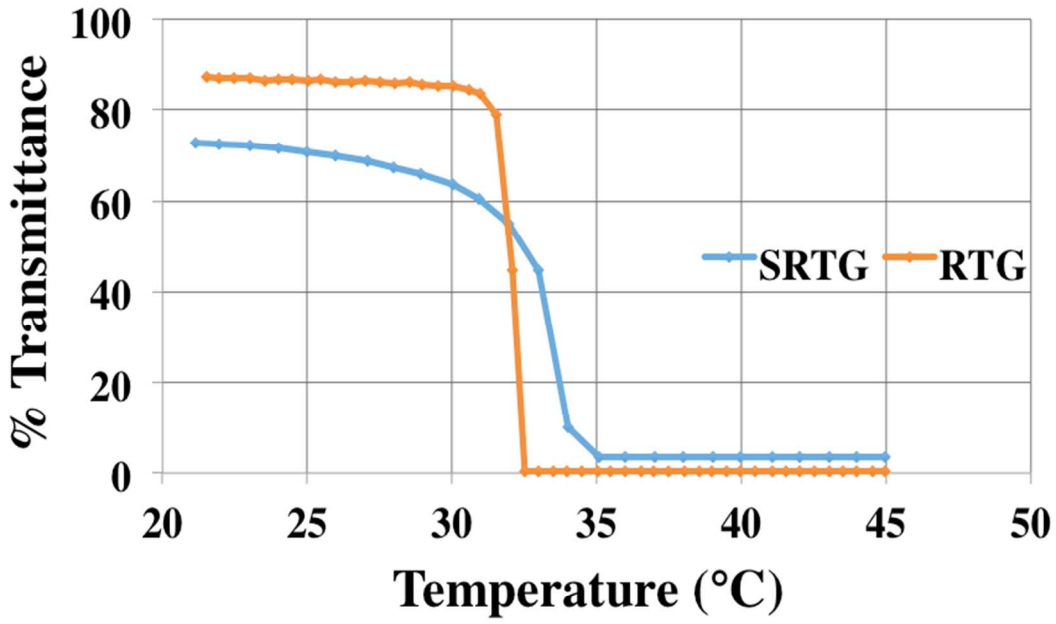


Fig S3. Temperature-dependent phase transition of SRTG (blue) and RTG (red). Both polymers display similar gelling temperature while the incorporation of the sulfonate groups to the SRTG slightly increased the gelling time.

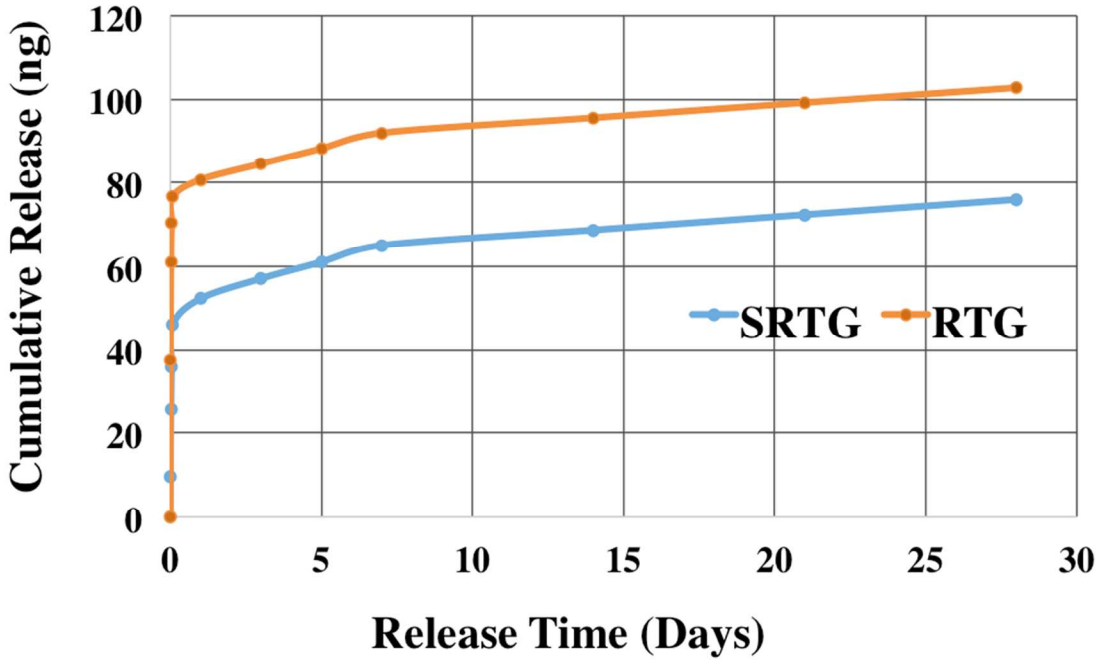


Fig S4. In vitro release profile of CNTF released from 1% SRTG and 1% RTG. Samples were taken over a month-long release test.

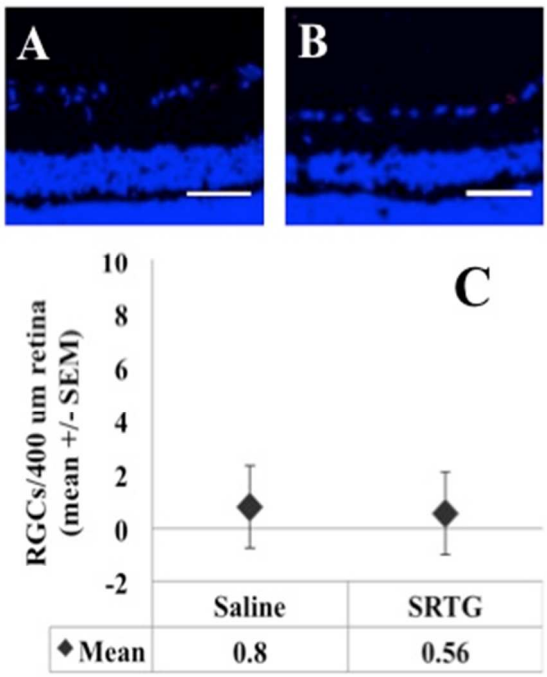


Fig. S5. Expression of Brn3a in retinal cross sections following ONC and Sul-PSHU-PNIPAAm intravitreal treatment after two weeks (B) when compared to a eye receiving ONC and saline injection (B). There was no significant difference observed between these two groups (C). Blue staining indicates DAPI. Red staining indicates Brn3a expression. Scale bar = 50μm.