

Supplementary Materials for “Effective Repair of Traumatically Injured Spinal Cord by Nanoscale Block Copolymer Micelles” by Yunzhou Shi *et al.***Synthesis and Characterization of Micelles.**

The mPEG-PDLLA di-block copolymer was synthesized by ring opening polymerization of D,L-lactide in the presence of mPEG as a macroinitiator and stannous 2-ethylhexanoate ($\text{Sn}(\text{Oct})_2$) as a catalyst¹. The ¹H NMR spectrum of the block copolymers was recorded on a Varian Unity Inova 500NB spectrometer (Palo Alto, CA, USA) operated at 500 MHz. Its chemical structure and ¹H NMR spectrum are shown in **Fig. S1**. The block length of PDLLA, 4,101 g/mol, was calculated from the peak intensity of CH_3 - (E, **Fig. S1c**) in comparison to the intensity of CH_2CH_2 - (B, **Fig. S1c**) of mPEG (MW 2,000 g/mol).

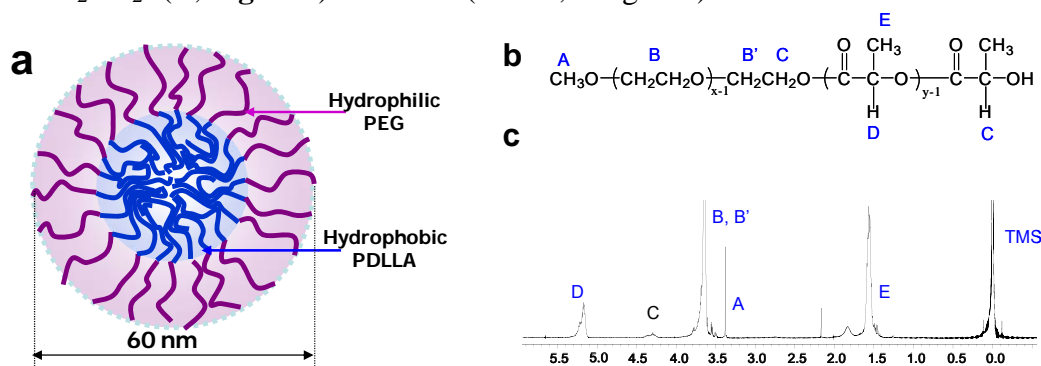


Figure S1. Structures of a mPEG-PDLLA block copolymer and its micelle. (a) Configuration of a D2K mPEG-PDLLA block copolymer micelle. Hydrophilic PEG chains are shown in purple and hydrophobic PDLLA block in blue. (b) Chemical structure of a mPEG-PDLLA block copolymer, A, B, B', C, D, E, are shown in the ¹H NMR spectrum. (c) ¹H NMR spectrum of mPEG-PDLLA block copolymer dissolved in CDCl_3 . The peaks are labeled according to the chemical composition. Reproduced from a paper by Shim *et al.*².

FITC-PEG-PDLLA(2,000:4,000) synthesis followed the same protocol as described previously³. Two solutions containing 0.4 g of H_2N -PEG- NH_2 , 50 μl of TEA, and 5 μl of DBDL in 5 mL of anhydrous dimethyl sulfoxide (DMSO) and 30 mg of fluorescein isothiocyanate (FITC) in 1 mL of anhydrous DMSO, respectively, were mixed and reacted at 80 °C for 4 h. DMSO and unreacted FITC were then removed against excess deionized water for 2 days at room temperature using a dialysis bag (MWCO 3,500, Spectra/Por RC), and FITC-PEG- NH_2 was purified by cation exchange column chromatography (CM Sephadex G-25, 1.5×30 cm column). Elution was performed with a linear NaCl gradient from 0 to 1 M (citrate buffer, pH 5.0). Fractions were collected, concentrated, and dialyzed against deionized water for 2 days. Yellow-orange powder was obtained by lyophilization. FITC-PEG-PDLLA was synthesized by coupling between FITC-PEG- NH_2 and PDLLA-COOH using *N,N*-diisopropylcarbodiimide, *N*-hydroxysuccinimide, and triethylamine.

The micelles were prepared by membrane dialysis². The mPEG-PDLLA copolymer (160 mg) was dissolved in 8 mL acetone. The block copolymer acetone solution was gently stirred for 30 min, and put through an atomizer to produce self-assembled micelles in 80 mL deionized water, followed by 4 h stirring. To remove the acetone completely, the solution was dialyzed against water for 24 h using a porous dialysis tubing (MWCO 3,500). Finally, the aqueous solution containing polymer micelles was filtered through a 0.45 μm -pored filter to remove undesirable

aggregates. A similar procedure was used to prepare mPEG-PCL (5,000:2,000) micelles, mPEG-PLLA (2,000:4,000) micelles and mPEG-PDLLA (5,000:5,000) micelles.

Size distribution of block copolymer micelles was measured by dynamic light scattering⁴ at 23°C on a Malvern PCS100 spectrogoniometer equipped with a LFI-1096 digital autocorrelator (DynaPro99, Protein Solutions, Inc.). The incident beam wavelength was 633 nm and the intensity autocorrelation was measured at a scattering angle of 90°. The CORAN software was used for data analysis to obtain the size distribution and the mean diameter.

The critical micelle concentration (CMC) was measured by monitoring the fluorescence of pyrene incorporated into the micelle core⁵. Aqueous solutions of mPEG-PDLLA copolymers at various concentrations from 1.0 to 5.6×10^{-6} mg/mL were mixed with a tetrahydrofuran solution of pyrene. Tetrahydrofuran was removed by stirring the solution at 40 °C for 4 h. The final pyrene concentration was 6×10^{-7} M. The spectral profile of pyrene was measured on a Varian Cary Eclipse Fluorescence Spectrophotometer equipped with a R3896 detector. The CMC was measured to be 1.4 µg/mL, equivalent to 230 nM, which was about 2,000 folds dilution of the original micelle solution. The micelle diameter was around 60 nm, as measured by dynamic light scattering. The micelles were found to be stable in size when kept at 4 °C for 3 months. The mPEG₅₀₀₀-PCL₂₀₀₀, mPEG₂₀₀₀-PLLA₄₀₀₀ and mPEG₅₀₀₀-PDLLA₅₀₀₀ micelles were synthesized using the same methods

Isolation of Spinal Cord White Matter.

Adult female guinea pigs were first anesthetized by 60 mg/kg ketamine hydrochloride, 0.6 mg/kg acepromazine maleate, and 10 mg/kg xylazine, then perfused with oxygenated, cold Krebs' (15°C) solution. The composition of the Krebs' solution was as follows (in mM): 124 NaCl, 2 KCl, 1.2 KH₂PO₄, 1.3 MgSO₄, 1.2 CaCl₂, 10 dextrose, 26 NaHCO₃, and 10 sodium ascorbate, equilibrated with 95% O₂ and 5% CO₂. The vertebral column was rapidly excised and the laminae from the lumbosacral to the cervical levels were removed in a continuous strip by cutting through the pedicles on either side. The roots on either side were cut carefully as the cord was gently removed from the inverted vertebral column and placed in cold Krebs' solution. The extracted spinal cord was first split into two halves by sagittal division and then cut radially to separate the ventral white matter from the grey matter. The ventral white matter was cut into 4-cm and 1-cm long strips. These strips were subsequently incubated in fresh Krebs' solution at room temperature for 1 h prior to the experiments.

Recording of CAP.

CAP was measured using a double sucrose gap recording chamber (**Fig. S2**). A 4.0 cm long strip of isolated guinea pig spinal cord white matter was supported in the central compartment and continuously perfused with oxygenated Krebs' solution (~2.0 mL/min) at 37°C maintained in a water bath. The free ends of the spinal cord strip were carried through the sucrose gap channels to side compartments filled with isotonic (120 mM) potassium chloride. The white matter strip was sealed on either side of the sucrose gap channels, using fragments of plastic coverslip and a small amount of silicone grease to attach the coverslip to the walls of the channel and seal around the tissue. Isotonic sucrose solution (230 mM) was continuously running through the gap channels at a rate of 1.0 mL/min. The axons were stimulated and compound action potentials were recorded at opposite ends of the strip of white matter by silver/silver chloride wire electrodes positioned within the side chambers and the central bath. Stimuli, in the form of

constant-current unipolar pulses of 0.1 ms duration, were adjusted to the smallest amplitude that could produce a full action potential for each sample.

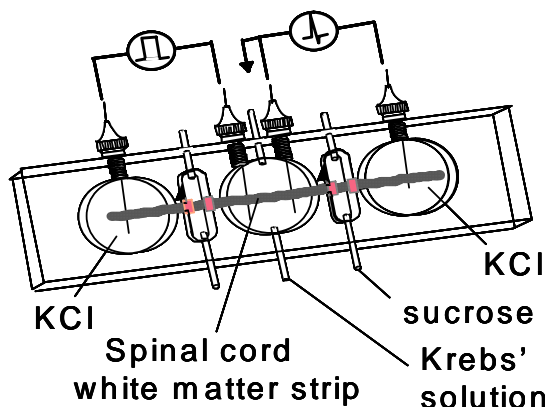


Figure S2. Schematic of the double sucrose gap recording chamber.

Multimodal Nonlinear Optical Imaging of Spinal Tissues.

The setup is shown in **Fig. S3**. The CARS signal was generated by two mode-locked 2.5-ps Ti:sapphire oscillators (Mira 900, Coherent Inc, Santa Clara, CA) with tunable wavelength from 700 nm to 1000 nm and tightly synchronized (Sync-Lock, Coherent Inc) with an average timing jitter of 100 fs. The laser beams were collinearly combined by using a dichroic combiner (LWP - 45 - R720 - 7850 - PW - 1004 - UV, CVI Laser LLC, Albuquerque, NM, USA) and directed into a laser scanning confocal microscope (FV300/IX70, Olympus America Inc, Melville, New York). A Pockels cell (Model 350-160, Conoptics, Danbury, CT, USA) was used to lower the repetition rate from 77 MHz to ~ 3.8 MHz. A 60X water objective with a 1.2 numerical aperture (Olympus Inc) was used to focus the excitation beams into the sample. The epi-detected (E-CARS) signal was collected by the same water immersion objective.

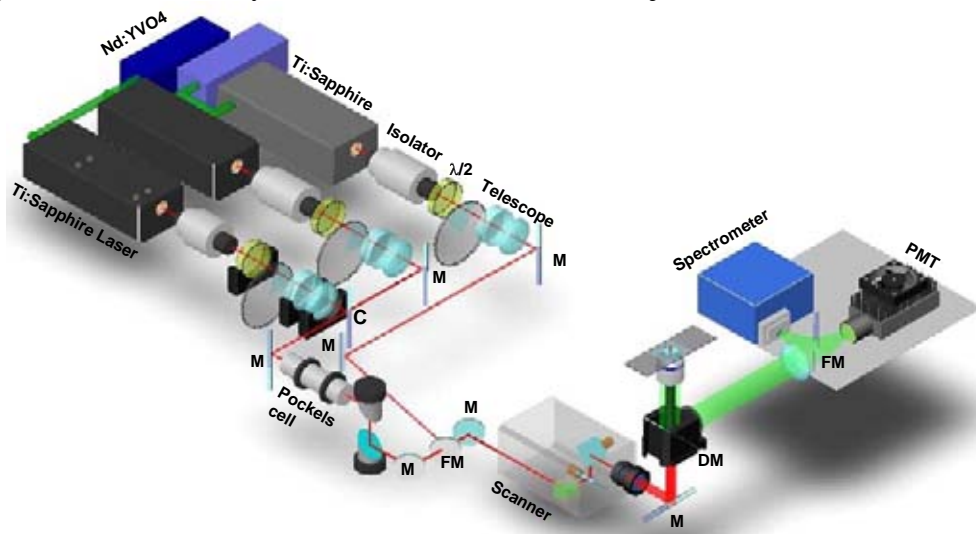


Figure S3. Schematic of a multimodal multiphoton microscope that allows CARS, SHG, TPEF imaging and spectral analysis on the same platform. C: combiner, DM: dichroic mirror, FM: flip mirror, M: mirror, PMT: photomultiplier tube.

A mode-locked femtosecond Ti:Sapphire oscillator (Mira 900, Coherent) was used for epifluorescence TPEF imaging of micelles. The 200 fs beam with a repetition rate at 77MHz was tuned to 735 nm and directed into the same laser scanning microscope. Both TPEF and E-CARS signals were detected with a photomultiplier tube (PMT, H7422-40, Hamamatsu, Japan) mounted at the back port of the microscope.

For monitoring calcium entry into axons, the spinal sample was pre-incubated in Ca^{2+} free Krebs' solution for 30 min, followed by Ca^{2+} free Krebs' solution with 40 μM Oregon Green 488 BAPTA-2 AM (Sigma, St. Louis, MO, USA) for 2 h. After that, the control group of healthy spinal cords was incubated in normal oxygenated Krebs' for 1 h; the control group of injured spinal cords was compressed and then incubated in normal oxygenated Krebs' for 1 h; the micelle treated group was compressed and then incubated for 1 h in oxygenated Krebs' solution supplemented with 0.67 mg/mL micelles. The TPEF signal of Oregon Green was transmitted through two 520/40 bandpass filters (Ealing Catalog Inc., Rocklin, CA, USA) and detected by an external photomultiplier tube (PMT) (H7422-40, Hamamatsu Corp., Bridgewater, NJ, USA). FluoView software (Olympus, Tokyo, Japan) was used to merge the TPEF and CARS images. TPEF intensities were determined by subtracting the background contributed by PMT dark current from the images using a FluoView software. A minimum of 55 axons were measured to obtain the average TPEF intensity of intra-axonal Oregon Green in each group. The intra-axonal space was identified by CARS signal of myelin sheath.

Presence of FITC Conjugated Micelles at the Injury Site.

One mL of 1.8 mg/mL micelles in saline made of FITC-PEG-PDLLA was injected into the rats immediately after spinal cord compression injury through tail vein. The rat was sacrificed 6 h post injection by pneumothorax. The spinal cord at the lesion site was extracted for confocal fluorescence imaging. Inside the injured tissue, we observed green fluorescence signals in the form of punctuated dots and fibrous structures (**Fig. S4a**), which were not observed in the tissue without injection of the micelles. The fluorescence signal was confirmed to arise from FITC-conjugated PEG-PDLLA copolymers based on the emission spectrum (**Fig. S4b**) recorded from the fluorescent spot in the image.

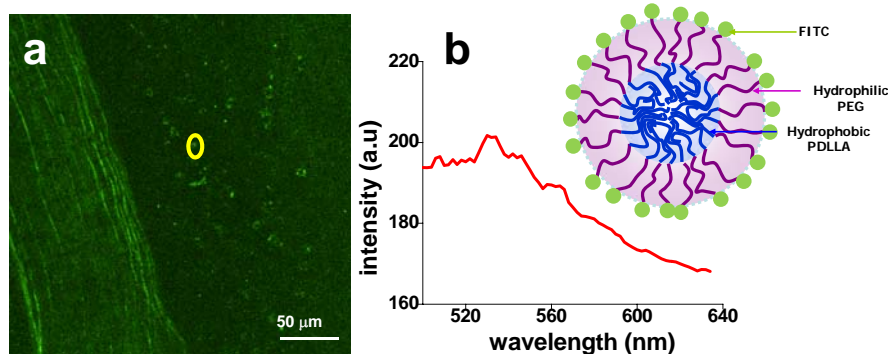


Fig. S4 (a) Confocal fluorescence image of an injured spinal tissue from a Long-Evans rat. The animal received a compression spinal cord injury and an immediate i.v. injection of 1.0 mL 1.8 mg/mL FITC-conjugated mPEG-PDLLA micelles. (b) The emission spectrum of the fluorescent spot in (a).

Cell Membrane Sealing in SCI Rats with Micelle Treatment.

To determine whether micelle treatment could repair the cell membrane in an injured spinal cord *in vivo*, we injected dextran-FITC, a fluorescent cell-impermeant dye with 4000 MWCO, into the cerebrospinal fluid of adult male rats prior to compression injury, and the anatomical location of cell bodies taking up the dye within 30 min following SCI was quantified^{6, 7}.

Animals were placed on an electric heating pad to maintain body temperature at 37°C during surgery. The rat was placed in a stereotaxic device and then the skull between the forehead and neck was exposed with a midline incision for both guide canulae. A hole was drilled at the 1.4 mm lateral and 0.8 mm posterior to bregma. The drill was then replaced with a guide canulae at 3.5 mm vertical into the lateral ventricle. An internal canulae connected to PE50 tubing was inserted into the guide canulae for injection. For the control group, 75 μ l of dextran-FITC (4 kDa molecular weight, 4% in PBS) (Sigma) solution was infused into the ventricle at 2 μ l/min rate using a graduated syringe. For the micelle group, 75 μ l of dextran-FITC solution with 1.8 mg/mL mPEG-PDLLA micelles was infused into the ventricle. The canulae was then allowed to remain in the ventricle for 10 min after completion of the infusion, after which the canulae was slowly removed. Gelfoam and suture were used to restore skull integrity. Next, 3 h post the infusion, the animal was prepared for spinal cord compression injury as described previously. The animal was then sacrificed through perfusion fixation 30 min post SCI. Cord segments approximately 1 cm in length, rostral and caudal to the T9-T11 segments where the lesion resides, were extracted and stored at 4 °C until use. The lesion tissue was cryostat-sectioned longitudinally at 35 μ m thickness and thaw mounted on slides. The slides were covered using an aqueous mounting medium. Images were obtained at 20X magnification using an Olympus IX70 confocal microscope (Olympus). Uptake of the membrane impermeable marker was quantified at 1-mm from the injury epicenter in both the rostral and caudal directions (n= 3 rats per group). Cells were counted using the ImageJ software (National Institutes of Health, Bethesda, MD). Only cells with neuronal morphology and sized from 4 to 100 μ m in diameter were included in the quantification in grey matter. Cell size in white matter was smaller, thus sizes from 4 to 25 μ m in diameter were included in the quantification in white matter. In order to reduce variability, two adjacent sections were counted at each side from the epicenter, and the cell numbers were averaged together to yield one data point per animal at each location. In the micelle treatment group, 1.8 mg/mL mPEG-PDLLA micelles were mixed with dextran-FITC and injected 3 h prior to compression injury.

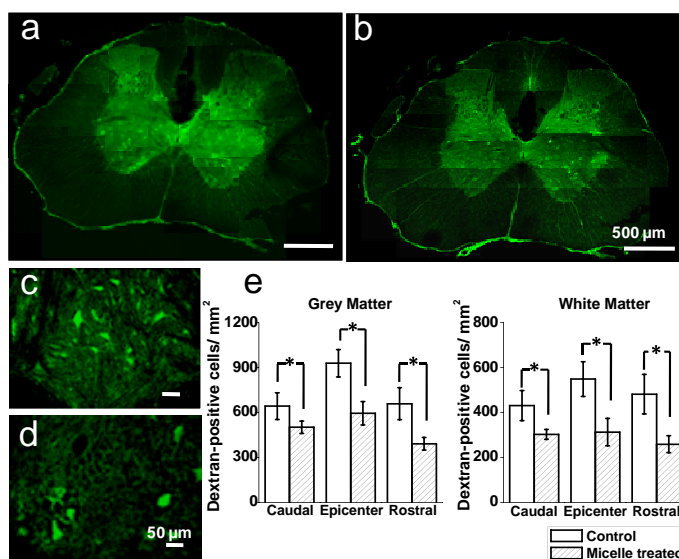


Figure S5. (a,b) Confocal fluorescence images of injured spinal tissues located 1 mm caudal from lesion epicenter in a control rat with saline injection after compression injury (a) and a micelle treated rat with 1.8 mg/mL mPEG-PDLLA micelles injected with dextran-FITC solution (b). (c,d) High magnification images show the representative cell density in grey matter in control (c) and micelle treated (d) spinal tissues, respectively. (e) Statistical analysis of dextran-FITC positive cell numbers in grey matter and white matter. * $p < 0.001$.

We assessed the cellular uptake of the membrane permeability marker at 1 mm caudal and rostral from the lesion epicenter as well as the lesion epicenter. The results are shown in Fig. S5.

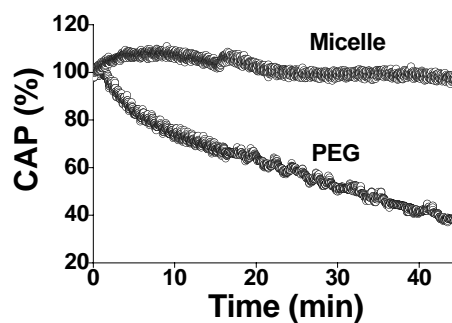
The density of dextran-positive cells was 502 ± 41 , 595 ± 78 , 391 ± 42 for the micelle treated group and 643 ± 89 , 929 ± 92 , 665 ± 107 for the control group with saline only at caudal, lesion epicenter and rostral in grey matter, respectively. While in white matter, the density of dextran-positive cells was 303 ± 22 , 313 ± 61 , 259 ± 38 for the micelle treated group and 431 ± 67 , 549 ± 77 , 482 ± 88 for the control group at caudal, lesion epicenter and rostral, respectively. Statistical analysis demonstrated a significant difference between the micelle group and the control group. These results demonstrate that the copolymer micelles could repair cell membranes in the spinal cord injured rats.

Histological Assessment and Calculation of Lesion Volume.

At week 4, animals were anesthetized (90 mg/kg ketamine and 5 mg/kg xylazine) and sacrificed by transcardial perfusion with cold PBS followed by 4% paraformaldehyde. Cord segments approximately 1 cm in length, rostral and caudal to the T9-T11 segments where the lesion resides, were extracted and stored at 4 °C until use.

The lesion tissue about 1 cm long was cryostat-sectioned longitudinally at 25 μm thickness and serially thaw mounted on slides. About 100 slices were prepared from each cord. 50 sections were immunoprocessed with glial fibrillary acidic protein (GFAP) to detect reactive astrocytes and the other 50 sections were stained with ED-1 to detect activated microglial cells and macrophages. In brief, all the slices were firstly permeated in 0.01M PBS (pH 7.3) containing 0.5% Triton X-100 (Sigma) for 5 min, then blocked in 0.01M PBS containing 0.1% Triton X-100 and 10% goat serum for 30 min, followed by incubation with polyclonal rabbit antibody against GFAP (diluted 1:220; Chemicon, Temecula, CA, USA) or mouse anti ED-1 monoclonal antibody (diluted 1:50; Millipore, St. Charles, MO, USA) overnight at 4 °C. The corresponding secondary antibody, biotinylated anti-rabbit IgG (H+L) (Sigma), was applied for 1 h at room temperature to locate the primary antibody. Sections were dehydrated and mounted with DPX mountant (Sigma) before evaluation. The lesion area of each section was visualized, outlined, and quantified using an Olympus IX70 confocal microscope equipped with a FluoView program. A 488 nm Argon laser, 20 mW before the microscope, was used to excite the fluorophore. Measurements were performed using a 4 \times objective. The total volume of the lesion was calculated by summing the individual subvolumes, calculated as the product of lesion area and slice thickness. High magnification (60 \times) was used to visualize detailed morphology of the reactive astrocytes and ED-1⁺ cells.

Figure S6. CAP recordings in healthy spinal cord strips circulated 45 min with a Krebs' solution supplemented with 0.67 mg/mL micelles or 50 wt% PEG. No significant CAP decrease was observed in the micelle-treated cord, but a decrease to 40% was observed in the cord treated by 50 wt% PEG.



Statistical analysis.

Independent experiments using spinal cords from different animals were performed to determine the average values and standard deviations (SD), expressed in mean \pm SD. The Student's *t*-test was used to compare the control and micelle-treated groups. The ANOVA Tukey's test was used for significance comparison between multiple groups.

References

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