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Repairing the Damaged Spinal Cord and Brain with Nanomedicine**

Youngnam Cho, Riyi Shi, Richard Borgens, and
Albena Ivanisevic**

There is no medical therapy for severe spinal-cord or brain injury that can restore behavioral loss in the chronic condition, or rapidly repair the membranes of damaged nerve cells in the acute stage of the injury. The latter permits rapid recovery of physiological functioning after injury, and largely vitiates continuing and progressive cell death. Here we describe for the first time a microcolloid composite, ranging from 50 to 300 nm, made of nonbiological, inert, and nontoxic components that fulfill all of the requirements of the latter therapy. From an applied-engineering standpoint, tools fabricated by nanotechnology have the potential to lead to more effective ways to treat and predict disease,[1,2] though a particular therapy for central nervous system (CNS) injury or disease has yet to be realized. Recent activity in nanotechnology has substantially improved colloid-based systems. The versatility of materials with inherent unique properties (optical, electrical, magnetic, and chemical) can be realized with the incorporation of a variety of biocompatible and biodegradable materials such as synthetic or natural polymers, lipids, or solid (metal, semiconductor, magnetic, or insulator) components. Of these, silica particles have several advantages: i) they ride upon a wealth of well-established methods for the synthesis and incorporation with other substances through surface modification and bioconjugation; ii) they have great potential to perform multifunctional activity; and iii) they exhibit intrinsic hydrophilicity, biocompatibility, and nontoxicity.[3–5] In addition, inorganic cores, rather than organic cores such as micelles, have a longer "shelf life".

[*] Dr. Y. Cho, Prof. R. Shi, Prof. R. Borgens
Center for Paralysis Research
School of Veterinary Medicine
Purdue University, West Lafayette
IN 47907 (USA)
E-mail: cpr@purdue.edu
R. Shi, Prof. R. Borgens, Prof. A. Ivanisevic
Weldon School of Biomedical Engineering
Purdue University, West Lafayette
IN 47907 (USA)
E-mail: albena@purdue.edu
Prof. A. Ivanisevic
Department of Chemistry
Purdue University, West Lafayette
IN 47907 (USA)

Here we describe the fabrication and characterization of polymer-surfaced silica colloids (PSCs; Figure 1), that are able to restore anatomical structure and physiological functioning to a severely injured mammalian spinal cord. This is accomplished through PSC surface-coat interaction with the nerve processes (mainly axons) of the damaged membranes. This interaction leads to spontaneous reassembly of the compromised membrane in this region, anatomical sealing, and the immediate recovery of nerve impulse propagation along the length of adult Guinea-pig spinal-cord white matter.[6] This was undertaken using well-established synthesis of colloids, functionalized for a chosen application, which was to specifically target nerve-membrane repair using poly(ethylene glycol) (PEG). Thus, our hypothesis was that specific targeting of damaged spinal cord white matter by PEG-coated particles, coupled with the polymer's capability to repair damaged neuronal membranes, would produce functional recovery. Anatomical integrity of the axons of spinal-cord white matter was assayed using dye-exclusion tests, and functional recovery was defined as the ability to rescue the ability of axons to conduct nerve impulses (action potentials) through the damaged region of the cord (see below). Using a colloid-based derivative, tunable concentrations of membrane-active molecules on their surfaces permits a significantly improved ability to control concentration and density of the polymer at the specific regions of CNS damage.

Figure 1A–C shows the synthesis of the PSC on the basis of Stober methods.[7,8,9] Figure 1D–F shows transmission electron microscopy (TEM) images of silica particles, where D shows bare silica particles, and E and F represent PEG-coated particles with different sizes: 50 nm and 400 nm, respectively. PSCs were fully characterized and their size distribution, chemical functionality, and type of ligand bonding were confirmed (D ∼ H).

The biological activity of PSCs was tested by i) evaluating the loss and recovery of electrophysiological conduction of nerve impulses through isolated Guinea-pig spinal-cord white matter, and in vivo tests using fully adult Guinea pigs with crushed spinal cords; and ii) determining the ability of spinal-cord tissue to reconstitute and seal damaged neuron/axon membranes against the uptake of extracellular labels or the loss of endogenous macromolecules from their cytoplasm. These functional assays are mutually dependent.[10]

Conduction through the length of an entire Guinea pig cord (≈40 mm) was studied in a novel double sucrose gap isolation/recording chamber[11] (Fig. 2A). This technique provides the most sensitive and highest-resolution recordings on ex vivo spinal cord or peripheral nerve known. Strips of isolated Guinea pig ventral white matter bathed in a physiological Krebs solution were evaluated, as this region of the cord possesses the highest density of large, fast-conducting myelinated axons. The normal characteristics of conduction of compound action potentials (CAPs or nerve impulses) were first recorded, and subsequently the cord was crushed in the mid-thoracic region using a laboratory-fashioned device. This possessed a détente to standardize compression between cords.[12] This procedure
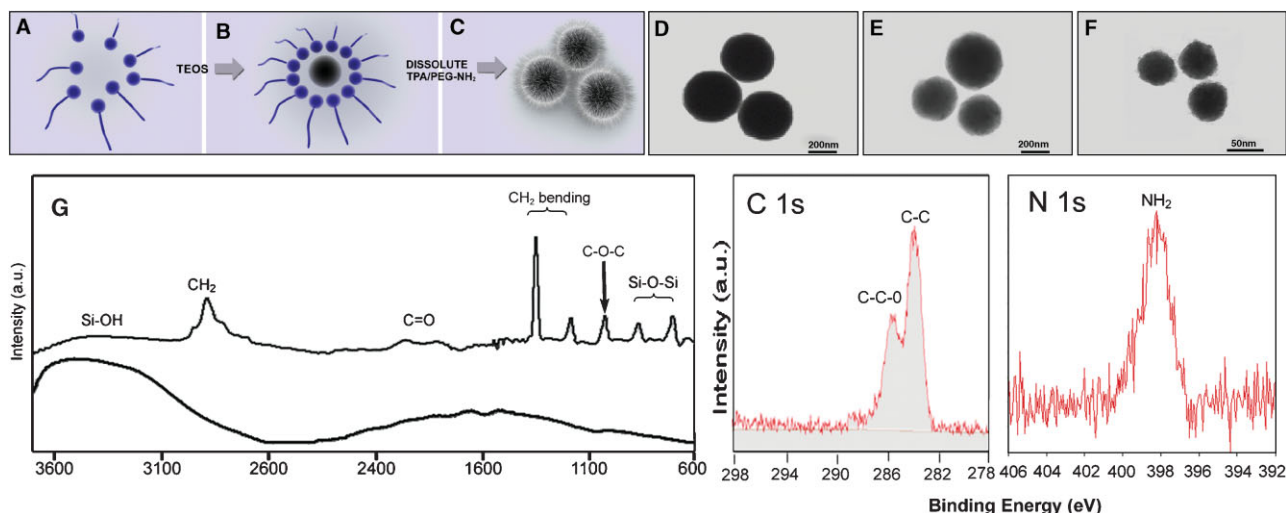
Figure 1. Silica colloid fabrication and characterization. We prepared silica colloids by combining water-in-oil (W/O) or reverse microemulsion methods with sol-gel technology (A–C). This process was performed at room temperature by hydrolysis of metal-organic compound, followed by post-coating with PEG. The emulsion solution comprising 1.77 g of Triton X-100, 1.6 mL hexanol, 7.5 mL of cyclohexane, 400 μL of water, and 100 μL of aqueous ammonia was stirred for 1 h at room temperature; subsequently 100 μL of tetraethyl orthosilicate (TEOS) was added. After a 24 h reaction, nanoparticles were separated from the emulsion solution by centrifugation and washed several times with ethanol and water. Subsequently, silica particles were reacted with a 1% ethanol solution of 3-(trimethoxysily) propyl aldehyde, (TPA) under nitrogen atmosphere for 6 h. The resulting aldehyde-terminated particles were further reacted with PEG-NH$_2$ ($M_W = 3000$), forming a covalent bond. The change of volume ratio of TEOS and NH$_4$OH had directly influenced the diameter of the particle. Transmission electron microscopy (TEM) showed that the freshly prepared colloids are approximately 400 nm in diameter and monodisperse, indicating regularity in size, shape, and structure (D). In addition, a dense layer of PEG polymers is coated on the surface of the particles by polycondensation with PEG (E and F). In (G) the record on the left is of Fourier transform infrared spectroscopy. The top record of this pair shows coated particles, the bottom record only bare silica particles. Reading left to right, PEG is shown at various intensities in the top record. The ordinate is in arbitrary units, the abscissa is in wavenumbers (cm^{-1}). The next two records show the results of X-ray photoelectron spectroscopy, used to monitor the chemical composition of synthesized colloids. The abscissa is binding energy in electron volts. The record to the left is a carbon record, and to the right a nitrogen record, of coated particles. These components are important in evaluating the chemical structure of the species coating the surface of the silica particles. After adsorption of PEG-NH$_2$, the C1s spectrum shows two marked peaks revealing C–C–C (284.5 Ev) and C–C–O (286 Ev). In addition, the monocomponent shape of the N 1 s spectrum (398 eV) further confirms the presence of chemically adsorbed PEG molecules.
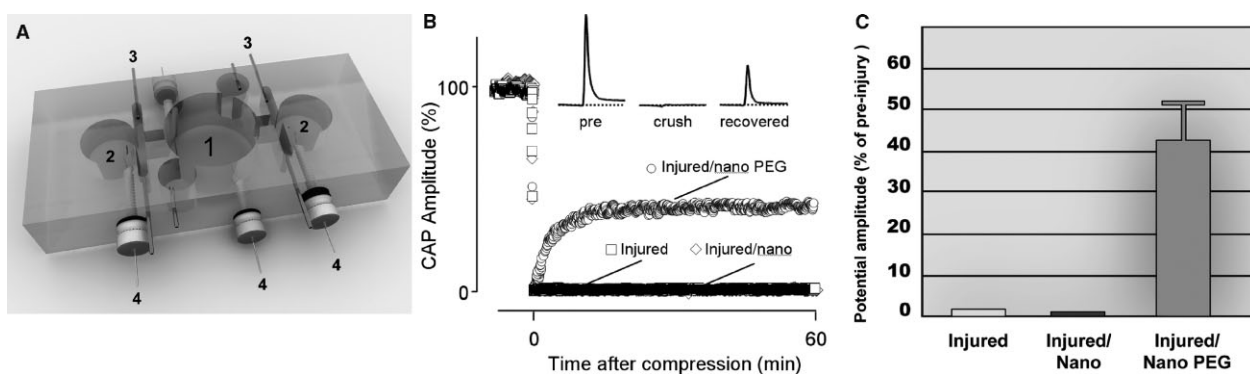

Figure 2. Physiological recovery of nerve impulses in crushed spinal cord with PSCs. In (A) a double sucrose gap isolation and recording chamber is shown. A strip of Guinea pig white matter (the length of the cord) is placed across all five chambers. The ends of the cord are electrically isolated by flowing boundaries of sucrose between the reservoirs of KCl and Krebs solution. CAPS are stimulated on one end of the white matter strip, and recorded at the other. In (B) three spinal cord records are shown from left to right: i) a typical CAP (pre-injury), followed by ii) its immediate elimination after compression in the center of the strip; iii) shows the recovery of the CAP, 60 min after topical application of 5 mM solution of PSCs in distilled water (see text). Crushed cords do not spontaneously recover CAPs at this time. Below these records, graphs of CAP amplitude vs. time show the responses of individual strips, injured but not treated, and PMSC treated. The average responses and their SEMs are shown for all 21 spinal cords in the bottom histogram (C, 7 cords in each group). The absence of any measurable CAP recovery is shown for silica-treated, while a miniscule recovery is shown for the uninjured cords due to one outlier (see text). Note the striking recovery of CAP propogation following treatment with the PEG microspheres. ($P \leq 0.001$).
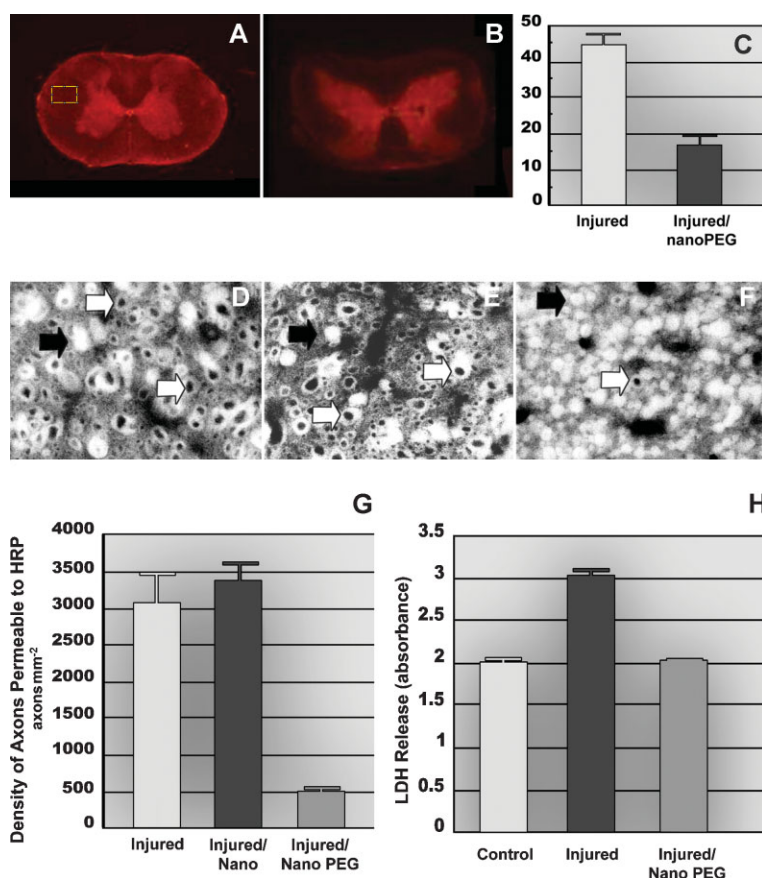
Figure 3. Anatomy of sealing in spinal-cord white matter. A and B) cross sections of spinal cord are shown in darkfield fluorescent illumination. The cords were exposed to TMR (1000 daltons) for 10 minutes prior to fixation and 4 h before sectioning (60-μm specimen on a freezing microtome). Cords were exposed to a solution of PSCs after a complete transection but prior to staining with TMR for 15 min. Untreated cords were treated similarly; (A) shows an injured untreated cord. Note the fluorescence in white matter associated with TMR uptake. This labeling was strikingly and statistically reduced by PSC exposure (the yellow inset shows the sample region for each pair of cords using NIH Image software). A total of 14 cords were used, treated as 7 pairs; one experimental (PSC treated) and one control untreated. For every cord, three records of intensity within the sample region were recorded within both left and right funuclii and the ventral funuclus. Approximately 150 μm outside of the cord, and directly adjacent to the original sample region, three similar records were obtained to record background fluorescence. The difference between these is displayed in arbitrary units (1–250 scale). A larger marker for membrane compromise (Horse-Radish peroxidase; HRP; 40 000 kD), was significantly imbibed into large numbers of crushed axons in (D). Each test solution was applied for ≈2 minutes, and the cord removed from the test chamber and fixed within 20 minutes. The white "halo" around each darkly label axon cross section is the myelin sheath (E and F). In (E) a similar level of labeling is shown after treatment with uncoated microcolloids. In (F) PSCs produced a striking and statistically significant reduction in membrane damage as revealed by HRP exclusion (G). Another indicator of membrane damage is the leakage of the enzyme, lactic dehydrogenase (LDH; 144 KD) into the extracellular milieu. Testing is accomplished by incubating crushed spinal cords or control spinal cords in Krebs solution for about 1 h, collecting the supernatant, and determining LDH concentration by spectrophotometric techniques. (H) shows the background level of LDH loss (enhanced by dissection, handling, and natural extrusion of the enzyme). Crush injury significantly increased LDH in the supernatant, while treatment with PSCs returned the level of LDH loss to pre-crush levels.

eliminated nerve-impulse conduction through the white matter (Fig. 2B). The recovery of conduction, or the lack of it, was then recorded i) in control untreated crushed cords; ii) after the addition of "control" silica particles without a PEG surface; and iii) after the addition of colloids possessing a polymeric surface coat. These samples were suspended in distilled water and added to the physiological medium in the central chamber. With only a single exception, only those spinal cord strips treated with PSCs were electro-physiologically competent within one hour of compression injury (Fig. 2B and C). Using these same techniques, spontaneous recovery of compound action potentials after compression rarely occurs; even then at times greater than one hour after injury. In one untreated spinal cord of seven, we observed the appearance of low-amplitude, weak, and unstable CAPs. In all cord treated with PSCs, statistically significant CAPs of marked amplitudes reappeared within five minutes. These initial CAPS then increased in magnitude over the one hour of observation. (Figure 2B and C). Uncoated colloids had no effect whatsoever on electrophysiological conduction. PSCs' ability to recover nerve-impulse conduction rapidly (in minutes) is based on the initial membrane-sealing ability of the polymer coating. The loss of potassium from within damaged axons at the exact site of damage is sufficient to extinguish the conduction of nerve impulses – thus even marginal resealing of this potassium "leak" can recover the neuron's ability to propagate nerve impulses.

Anatomical sealing in whole spinal cords isolated to Krebs solution was tested by application of the fluorescent label tetramethyl rhodamine dextran (TMR; 10 kD) to the bathing solution after complete transection of the whole spinal cord. This label is imbibed into damaged neurons and their processes. Since the behavioral loss in animals and man after spinal cord injury (SCI) is due to the failure of conduction though white matter - and not the loss of neurons[13–14] - only white matter was evaluated in these studies. In transected spinal cords, the labeling of white matter was quantified using NIH Image software (Fig. 3A and B). Untreated white matter of all severed cords was well labeled, while the labeling of PSC-treated spinal cords was barely detectable. Quantification of fluorescence (minus background) statistically confirmed what was easily observed in paired treated and untreated cords ($P \leq 0.01$; Mann-Whitney, two-tailed test; Fig. 3C.). This result suggested the recovery of electrophysiological conduction was tied to rapid sealing and sparing of the cell processes of white matter. This is an extreme "proof of concept"
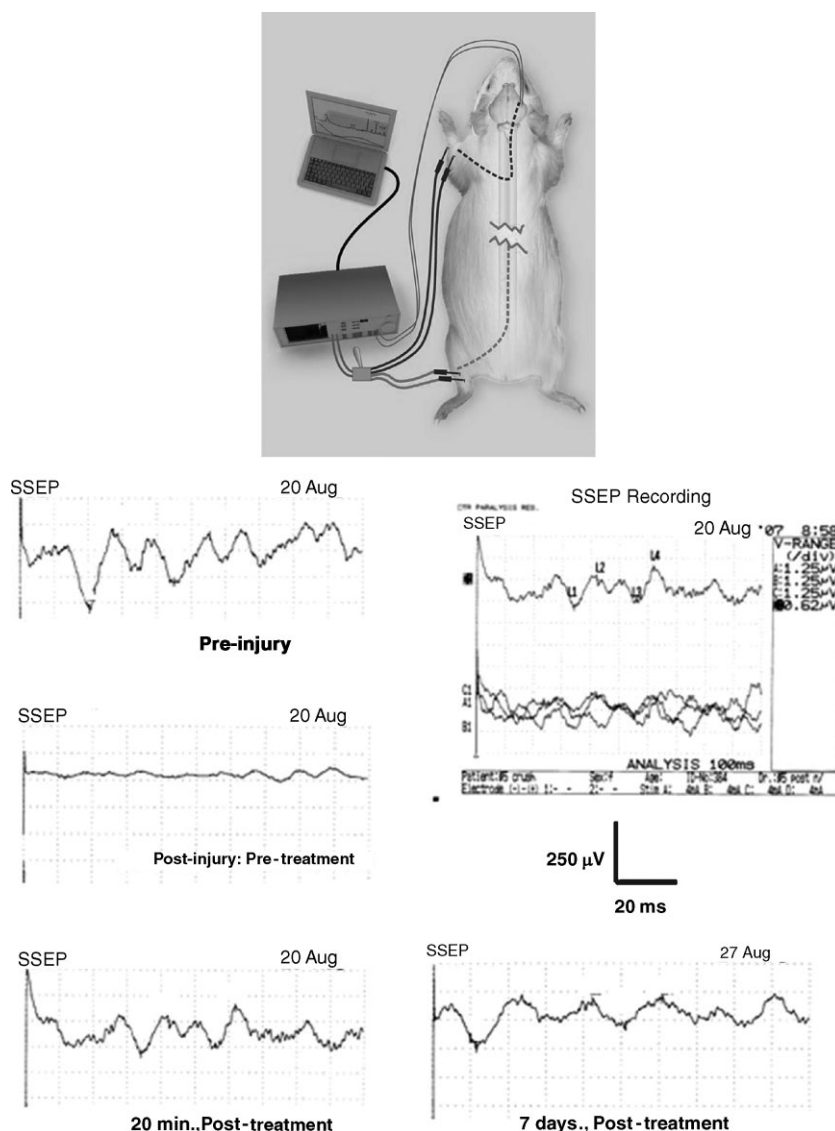
Figure 4. Rapid recovery of spinal cord conduction in vivo. The drawing at the top shows the stimulation and recording arrangement used to measure EPs crossing — or failing to cross — a mechanically crushed Guinea pig spinal cord in the midthoracic region. Note that the median nerve of the foreleg to the sensorimotor cortex pathway is above the level of the lesion and unaffected by it. Such control recording eliminates the possibility of "false negative recordings". Stimulation of either nerve of the legs is toggled between this pathway and the tibial nerve stimulation (of the hind leg) after recording electrodes are placed to record sensorimotor EPs from the contralateral side. Recording of EPs at the level of the brain generated by tibial nerve stimulation is eliminated by severe spinal cord compression in the midthoracic region. A sample of a complete SSEP recording is shown (top, second column), the bottom three individual recordings are trains of 200 stimulations ($\approx$3 mA square wave; 200 μs duration) at 3 Hz. These are averaged (single upper trace) using a Nihon Koden Neuropak 4. Pre-injury to 7 days post-injury records show only the averaged recordings in animal PSC 6. In the pre–injury recording, note up to five individual "peaks" representing early- and later-arriving EPs after stimulation. The complete elimination of these EPs after spinal cord injury is shown. A rapid and near complete recovery of both early ($\approx$40 ms latency) and later ($>$50/60 ms) arriving EPs after PMSC injection is evident, as is the stability of the recovered EPs with time. All animals injected with uncoated silica particles failed to recover any EP for the entire time of observation. Their records were identical to that shown for the post-injury yet pre-treatment recording. Typical SSEPs were elicited by median nerve stimulation in all subjects at all recording periods (data not shown).

test as complete transection of cords in clinical injuries is uncommon.[15–16]

The result of this "dye exclusion" test[17] was further confirmed by PSC-mediated exclusion of a larger label, horseradish peroxidase (HRP; $\approx$41 kD). HRP, when added to the bathing media of the injured spinal cord's heavily labeled damaged axons, is revealed as a dark brown/black axonal cross section framed by the wrapping of unstained myelin (Fig. 3D–F). This uptake was significantly reduced after treatment with PSCs. Similarly, the normal background loss of the cytoplasmic enzyme lactic dehydrogenase (LDH; 144 kD) to the extracellular milieu is significantly increased after damage to the axolemma.[12] Treatment with PSCs after injury substantially reduced this efflux to normal baseline levels (Figure 3H).

While particles with a diameter ranging from 250–400 nm were used in these initial evaluations, only 50-nm-diameter PSCs were suspended in injectable sterile Ringer's solution for live-animal testing. Appropriate institutional review of the animal protocol was conducted and an approval was received prior to any animal testing. It was impossible to predict if relatively large microcolloids might agglutinate, possibly producing a form of thrombus. Furthermore this unexpected outcome could not be identified in the ex vivo testing, since these spinal cord strips were evaluated without blood flow. Therefore the smallest range of PSC diameters was chosen for live-animal testing. This would have the additional benefit of increasing the potentially useful surface area of PSCs in contact with local, and perhaps very small, regions of cell-membrane defects.

Adult Guinea pigs ($\approx$400 gm) were anesthetized, their midthoracic spinal cord exposed by hemilaminectomy procedures and crushed using forceps possessing a détente as above. Somatosensory evoked potentials (SSEP) recordings were made by stimulating the tibial nerve of the hind paw, and recording the arrival of EPs approximately 40–50 ms later at the contralateral sensorimotorcortex. A control for "false negative" EPs was accomplished by stimulation of the median nerve of the forepaw, and "toggling" between these two neural circuits - the latter is rostral to the injury and unaffected by it (Fig. 4 inset). Electrophysiological recordings were made before spinal cord compression surgery, after surgery, within 15 minutes of subcutaneous

PSC injection, and at 1 day and 1 week post treatment. All eight of the animals injected with PEG-coated particles recovered SSEPs no later than 15–20 minutes after injection. Moreover, the rough timing and amplitude of both early- and late-arriving EPs after stimulation were consistent with baseline recordings (pre-injury records; Fig. 4) None of the 5 animals injected with uncoated particles recovered SSEPs for observation times of 1 week. This result was strikingly significant ($P = 0.0008$; Fishers' exact test).

An additional and important property of the PSC composites is their preferential targeting of damaged CNS, likely due to their strong hydrophilia.[18–19] This was revealed by incorporating a label into the composite and comparing its distribution between crushed and undamaged spinal cord (data not shown). These results provide the first evidence for substantial neuroprotection, correlated to a physiological recovery in significantly damaged CNS tissue, using colloid technology. This technique is based on the engineered surface properties of the colloid, which is not typical of pharmacological covalent bonding. These colloids are unable to be metabolized (as is the case with drugs) and would be injected into the patient's bloodstream to be harmlessly passed out of the body, as both native components are inert and/or nontoxic.[20–21]

For over 30 years hydrophilic polymers such as PEG have been used to fuse cell membranes. Fusion of defective cell membranes with PEG or copolymers such as the poloxamines has been shown to vitiate or alleviate conditions as diverse as burns, myonecrosis, and testicular reperfusion injury in animal models.[6,13] The literature describing membrane, and model membrane, fusogenic responses to hydrophilic polymers and non-ionic surfactants (fusogens) has been reviewed elsewhere.[22–24] Briefly, it is believed the strong hydrophilic character of the polymer dehydrates local regions of membrane compromise - targeted by the relative hydrophobic nature of these defects relative to surrounding "normal" membrane. It is likely that the initial sealing and subsequent fusion involves the architecture and organization of water at the membrane and molecular level. Relative dehydration of the membrane defect is thought to allow the "interrupted" central lipid core of the membrane (strongly hydrophobic) to then resolve back together, thus erasing the defect. Eventual loss/reduction of the polymers lead to a rehydration of the inner and outer lamella, and the reorganization of various proteins and receptors, whose organization within the plane of the membrane is in part dependent on the polar forces of the hydrated layers. However, it is prudent to remember these possible molecular mechanisms of membrane fusion/reassembly produced by certain polymers are still being investigated using model membranes. The fusogenic mechanisms of hydrophilic polymers are not entirely understood, but the responses to their application to membranes is very clear, and has formed the basis of cell fusion methods used to produce monoclonal antibodies for the past 30 years. The safety of PEG has been reviewed by others.[21] Importantly, hydrophilic polymers such as PEG and poloxamer 1100 are thought to be safe for human application.[21] Moreover, we have shown that injection of ≈ 2000–$3000\,M_W$ PEG or Poloxamer 1100 in both adult Guinea pigs and rats can produce anatomical sealing of damaged neuron membranes and restore their conduction properties in hours to days.[10,24] Anatomical repair and functional recovery occurred in both spinal-cord-injured and brain-injured Guinea pigs and rats.[19,25,26] This work has progressed through clinical trials in canine paraplegia.[27] Currently, we cannot increase either the molecular weight (M_W) of the polymer or its aqueous concentration in an effort to widen the therapeutic window and behavioral recovery after CNS damage. At higher molecular weight (M_W) or concentration, increasing viscosity limits its clinical utility. At lower M_W ($\approx < 1000\,\text{kD}$) there is a risk associated with dimers or trimers that continue to be circulated in the fluid compartments of the body and remain available for catabolism into monomers, producing ethylene glycol poisoning. Moreover, M_W in this range has limited capability to induce membrane fusion as well.[21]

In short, we have reached the safe limits for a clinical therapy based on injectable polymers suspended in clinically compatible fluids. The technology reported here permits precise, inert, and nontoxic particle fabrication possessing densities of surface-active agents (PEG) many orders of magnitude greater than can be achieved by conventional systemic administration. This also beneficially impacts the occurrence of concentration-dependent side effects. Moreover, PEG-coated particles are able to reduce the reticuloendothelial system (RES) uptake, resulting in long circulation time after intravenous administration. When compared with PEG molecules themselves, the use of nanoparticles substantially facilitates internalization of PEGs inside the cells, because nanometer-sized particles can easily penetrate across the membrane barrier by endocytosis. The internalization of PEGs is beneficial to recover abnormal mitochondria behavior induced by the endogenous toxin such as acrolein. Presently these PSCs, and another form - incorporating an antidote to the toxic products producing secondary injury after spinal cord or brain injury[28] - are undergoing testing in spinal cord and head injury models of these catastrophic injuries, and the beginnings of clinical testing in naturally injured paraplegic dogs at two cooperating university veterinary teaching hospitals (refer elsewhere[27] for a model).

Keywords:

central nervous system · colloids · medicine · nerve membranes

[1] G. Downing, *Science* **2005**, *310*, 1132–1134.
[2] E. Callaway, *Science* **2006**, *314*, 1674–1676.
[3] D. Acros, C. V. Ragel, M. Vallet-Regi, *Biomaterials* **2001**, *22*, 701.
[4] W. Zhao, J. Gu, L. Zhang, H. Chen, S. Shi, *J. Am. Chem. Soc.* **2005**, *127*, 8916–8917.
[5] L. Wang, *Anal. Chem.* **2006**, *78*, 646–654.
[6] R. B. Borgens, *Neurosurgery* **2001**, *49*, 370–379.
[7] R. Lindberg, J. Sjoblom, G. Sundholm, *Colloids Surf. A.* **1995**, *99*, 79–88.
[8] S. Santra, *Anal. Chem.* **2001**, *73*, 4988–4993.
[9] R. P. Bagwe, L. R. Hilliard, W. Tan, *Langmuir* **2006**, *22*, 4357–4362.
[10] R. Shi, J. D. Pryor, *Neuroscience* **2000**, *98*, 157–166.
[11] R. Shi, A. R. Blight, *J. Neurophysiol.* **1996**, *76*, 1572–1580.
[12] J. Luo, R. B. Borgens, R. Shi, *J. Neurotrauma* **2004**, *21*, 994–1007.

[13] R. B. Borgens, *Advances in Anatomy, Embryology and Cell Biology, (Monograph)*, Springer-Verlag, Heidelberg, Germany **2003.**

[14] A. R. Blight, *J. Neurosci.* **1991**, *103*, 156–171.

[15] R. P. Bunge, W. R. Puckett, J. L. Beccerra, A. Marcillo, R. M. Quencer, *Adv. Neurosci.* **1993**, *59*, 75–89.

[16] M. H. Tuszynsksi, K. Gabriel, K. Gerhardt, S. Szollar, *J. Neurotrauma* **1999**, *16*, 523–531.

[17] R. Shi, R. B. Borgens, *J. Neurocytol.* **2000**, *29*, 633–643.

[18] R. B. Borgens, D. M. Bohnert, *J. Neurosci. Res.* **2001**, *66*, 1179–1186.

[19] A. O. Koob, B. S. Duerstock, C. F. Babbs, Y. Sun, R. B. Borgens, *J. Neurotrauma* **2005**, *22*, 1092–1111.

[20] S. Jin, K. Ye, *Biotechnol. Prog.* **2007**, *23*, 32–41.

[21] P. Working, M. S. Newman, J. Johnson, J. B. Cornacoff, in *Poly(ethylene glycol) Chemistry and Biological Applications* American Chemistry Society, Washington DC, 2001, pp. 45–57.

[22] J. Lee, B. R. Lentz, *Biochemistry* **1997**, *36*, 6251–6259.

[23] N. Nakajima, Y. Ikada, *J. Biomed. Sci. Poly. Ed.* **1994**, *6*, 751–759.

[24] R. L. Davidson, P. S. Gerald, *Somatic Cell Mol. Genet.* **1976**, *2*, 165.

[25] R. Shi, R. B. Borgens, *J. Neurophysiol.* **1999**, *81*, 2406–2414.

[26] R. B. Borgens, R. Shi, *FASEB J.* **2000**, *14*, 27–35.

[27] P. H. Laverty, A. Leskouar, G. J. Breur, J. R. Coates, R. L. Bergman, W. R. Widmer, J. P. Toombs, S. Shapiro, R. B. Borgens, *J. Neurotrauma* **2004**, *20*, 1767–1777.

[28] P. Liu-Snyder, R. B. Borgens, R. Shi, *J. Neurosci. Res.* **2006**, *84*, 219–227.