# Neuroprotection from secondary injury by polyethylene glycol requires its internalization

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#### Summary

Polyethylene glycol (PEG) is well known to both fuse and repair cell membranes. This capability has been exploited for such diverse usages as the construction of hybridomas and as a reparative agent following neurotrauma. The latter development has proceeded through preclinical testing in cases of naturally induced paraplegia in dogs. The mechanisms of action of polymermediated neurorepair/neuroprotection are still under investigation. It is likely that the unique interaction of hydrophilic polymers with the mechanical properties of cell membranes in concert with an ability to interfere with mechanisms of secondary injury such as the production of highly reactive oxygen species (ROS or 'free radicals') is the basis for neuroprotection by polymers.

Here we provide further evidence that the ability of PEG to reduce or limit secondary injury and/or lipid peroxidation (LPO) of membranes requires entry of PEG into the cytosol, further suggesting a physical interaction with the membranes of organelles such as mitochondria as the initial event leading to neurorepair/neuroprotection.

We have evaluated this relationship *in vitro* using acrolein, a potent endogenous toxin that is a product of LPO. Acrolein can pass through cell membranes with ease, inducing progressive LPO in 'bystander' cells, and the production of even more acrolein by inducing its own

#### Introduction

Recently this laboratory, in collaboration with the Veterinary Clinical Sciences Department of Purdue University and the College of Veterinary Medicine at Texas A&M University, has shown that intravenous injections of an ~30% solution of polyethylene glycol (PEG; 3500 Da) in sterile saline can produce unexpected recovery of functions in naturally produced, severe, canine spinal cord injury. This occurred in neurologically complete, paraplegic dogs treated in a hospital setting (Laverty et al., 2004). Supportive laboratory animal data production. Immediate application of PEG (10 mmol l<sup>-1</sup>, 2000 Da) to poisoned neurons in vitro was unable to rescue them from necrosis and death. Furthermore, threeconfocal microscopy of fluorescently dimensional decorated PEG shows that it does not enter these cells for up to 2 h after application. By this time the mechanisms of necrosis are likely irreversible. Additionally, severe oxygen and or glucose deprivation of spinal cord white matter in vitro also initiates LPO. Addition of potent free radical scavengers such as ascorbic acid or superoxide dismutase (SOD) is able to interfere with this process, but PEG is not. Taken together, these data are consistent with the hypothesis that PEG is able to rescue mechanically damaged cells, based on a restructuring of the damaged plasmalemma. Furthermore, in compromised cells with an intact cell membrane, PEG must first gain access to the cytosol where this same capability may be useful in restoring the integrity of cellular organelles such as mitochondria, though the intracellular concentration of the polymer must be significant relative to the concentration of toxins produced by LPO in order to rescue the cell.

Key words: PEG, secondary injury, acrolein, endogenous toxins, CNS.

for initiating this preliminary clinical trial will be described below; however, the long record of safe usage of PEG in human medicine (Working et al., 1997) has pressed these experiments to the brink of human clinical trials in spinal cord injury (SCI) and traumatic brain injury (TBI) (Sofamor Danek/Medtronics Corporation).

Though the behavioral responses to injected and topically applied PEG in various models of neurotrauma are consistently positive (Shi et al., 1999; Shi and Borgens, 1999; Borgens and Shi, 2000; Borgens and Bohnert, 2001; Borgens et al., 2002;

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Koob et al., 2005; Koob and Borgens, 2006), the mechanisms of action underlying this recovery are incompletely understood (Borgens, 2003). The mechanical damage that cell membranes undergo indeed leads to collapse and death of the cell body at variable rates dependent on many factors, including the magnitude of the insult [for real time imaging using atomic force microscopy, see McNally and Borgens (McNally and Borgens, 2004)]. However, cells that survive this initial damage undergo a process of biochemically mediated, progressive collapse and death called 'secondary injury'.

Large hydrophilic polymers and non-ionic surfactants have shown promise as therapeutic agents in numerous progressive injury models as diverse as burns, electric shock myonecrosis, testicular reperfusion injury and spinal trauma (Padanilam et al., 1994; Lee et al., 1992; Lee et al., 1993; Palmer et al., 1998; Borgens and Shi, 2000). It is believed this ability to rescue cells and tissues from progressive destruction resides in the ability of these polymers to interact with regions of damaged membrane and to rapidly restore structural integrity to them.

This membrane 'sealing' reduces the exchange of ions and molecules across the plasmalemma and restores excitability in neurons and their processes within minutes of PEG application to damaged spinal cords in organ culture (Shi et al., 1999; Shi and Borgens, 1999). The biophysics underpinning the action of fusogenic polymers is still an active area of investigation using cells and model membranes (Lee and Lentz, 1997; Lentz, 1994; Borgens, 2003; Yasuda et al., 2005; Georgiev and Lalchev, 2004; Georgiev et al., 2006). In crushed nerves, PEG application significantly reduces or vitiates the uptake of labels such as horseradish peroxidase (HRP) and ethidium bromide applied to the extracellular mileau (Luo et al., 2002; Shi and Borgens, 2000; Koob et al., 2005). A marked increase in lactic dehydrogenase (LDH) escaping from damaged cells into the extracellular fluid *via* their compromised membranes is also typical of cell trauma. This LDH efflux is reduced by polymer application (Luo et al., 2002). Intracellular labels trapped within other types of cells such as myocytes are released into the extracellular environment in vitro after insult; however, this exodus is eliminated after exposure to PEG or Poloxamers (see Lee et al., 1993). These data support the membrane 'sealing' function of PEG producing a rapid recovery of the membrane's 'fence' properties. Strong circumstantial support is also provided by a recovery of conduction in crushed axons within minutes of exposure to aqueous PEG (Shi and Borgens, 2000).

Recently, the copolymer Poloxamer 188 (which is >80% PEG) has been described as a 'free radical scavenger' (Marks et al., 2001). The scavenging of reactive oxygen species (ROS) would be beneficial to the structural repair of damaged membranes. Mechanical damage, as well as ischemic episodes, in soft tissues leads to cell death and dysfunction through aberrant oxygen metabolism at the level of mitochondria. This is associated with the upregulation and liberation of ROS, and the interaction of these oxidative agents with the inner domain of cell membranes, producing potent toxins such as acrolein and hydrononeal *via* lipid peroxidation (LPO) (Liu-Snyder et al., 2006a).

Here we show that application of acrolein to the medium of PC12 cells in culture results in a rapid and significant destruction within 4 h. Furthermore this 'die-off' is dependent on the concentration of the poison (Liu-Snyder et al., 2006b). The most lethal concentration of acrolein tested (100 mol  $l^{-1}$ ) is still, however, at the high end of its measured physiological range (Nardini et al., 2001; Satoh et al., 1999). Since acrolein passes through membranes and is upregulated during the catabolism of membranes, a positive self-reinforcing feedback cycle helps to promote the destruction of tissues. Moreover, this cycle of tissue degeneration is reversible, since the mechanism of cell death following LPO is necrosis and not apoptosis (Liu-Snyder et al., 2006a).

We evaluated the application of PEG as a means to interfere with acrolein toxicity using PC12 cells in culture, and the ability PEG to enter these cells if required for this neuroprotection. Though PEG enters compromised cells *via* the membrane, it is unknown how permeable relatively intact cell membranes would be to the polymer. Secondly, we further explored this notion at the tissue level using isolated spinal cord white matter exposed to acrolein in a double-sucrose gap recording/isolation chamber (Shi et al., 2002; Peasley and Shi, 2003; Shi and Blight, 1996; Shi and Blight, 1997).

## Materials and methods

#### PC12 cell culture, differentiation

PC12 cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 50 i.u. ml<sup>-1</sup> of penicillin and 5 mg ml<sup>-1</sup> of streptomycin. Culture conditions were 5% CO<sub>2</sub> at 37°C. Cells were seeded at  $1 \times 10^6$  cells per dish or well. For neuronal differentiation, 10 ng ml<sup>-1</sup> of nerve growth factor (NGF) was present in the culture medium. Immunostaining of the cells began when all the cells had differentiated processes.

#### Application of acrolein and PEG exposure

Acrolein  $(10^4 \,\mu\text{mol}\,l^{-1})$  was made fresh in phosphatebuffered saline (PBS) as the stock solution and diluted to  $100 \,\mu\text{mol}\,l^{-1}$  or  $200 \,\mu\text{mol}\,l^{-1}$  just before adding to the cell culture medium. Polyethylene glycol (PEG) was made as a stock solution of 100 mmol  $l^{-1}$  and diluted to 10 mmol  $l^{-1}$  just before adding to the cell culture. PEG was applied 15 min after the addition of acrolein. In the control group, an equal volume of PBS instead of PEG was added to the medium.

# Cytotoxicity measurement (MTT assay)

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was reconstituted in PBS and added to each well 1 h before the termination of the experiment. Cells were seeded at  $1 \times 10^6$  cells per well in 12-well plates for at least 2 h before they were exposed to acrolein. Experiments were completed 4 h after the application of acrolein. After incubation, cells cultures were removed from the incubator. MTT solubilization solution was added to each well to dissolve the remaining formazan crystals. The absorbance of each sample was measured using Spectrophotometric (SPECTRA, SLT Lab Instruments, Salzburg, Austria) at 550 nm minus the background at 660 nm.

#### Confocal microscopy of fluorescently conjugated PEG

Attachment and movement of PEG was evaluated by using PEG with a covalent dansyl chloride decoration. Its ideal florescent properties are: emission range, 300-500 nm; excitation range, 200-500 nm. It is very unlikely that the dansyl group would enhance surface binding of PEG, but could possibly aid transport across the membrane, however, this was not tested in these investigations. Similar to PEG, the decoration has no net charge. To evaluate movement and attachment of PEG, differentiated PC12 cells were grown on glass coverslips. Cell cultures were stopped at 1 h, 2 h and 3 h after addition of acrolein to the culture medium. The coverslips were washed three times with PBS (pH 7.4) and switched to a buffered fixative (4% paraformaldehyde; v/v) for 20 min. The coverslips were washed three times for 5 min each with PBS. Following that, the coverslips were mounted on slides with Aquamount mounting medium and sealed using nail polish. Confocal microscopy images were acquired using a MRC-1024 (Bio-Rad, Hemel Hempstead, UK) on a Diaphot 300 (Nikon, Tokoyo, Japan) inverted microscope using a  $60 \times 1.4$  NA lens. The 488 nm line of the krypton-argon laser (Ion Laser Technology, Salt Lake City, UT, USA) was used to excite fluorescein-conjugated PEG and the emission was detected using a 515 nm long pass filter. Z-series images were collected using a 0.45 µm z-step motor. Projections and animated rotating projections were constructed using MetaMorph (Universal Imaging, West Chester, PA, USA) image software.

# Double sucrose gap recording: isolation and recording from long tract spinal cord axons

The spinal cord of the adult guinea pig was quickly dissected from deeply anesthetized animals, and placed in oxygenated Krebs' solution. Each strip of spinal cord ventral white matter (~38-40 mm) was produced using a fine blade and previously described techniques (Shi and Blight, 1997; Shi and Borgens, 1999; Peasley and Shi, 2003). These strips were ideal for recording of compound action potentials through the length of cord, due to the relative abundance of large caliber myelinated axons typical of this region. Strips were maintained in oxygenated Krebs' solution until placement in a double sucrose gap chamber. The strips were placed lengthwise across five separate chambers as described below. Precise details of the construction and use of double sucrose gap recording chamber has been described previously (Shi and Blight, 1997; Shi and Borgens, 1999; Peasley and Shi, 2003). Briefly, the device consists of three large reservoirs - roughly the same size and depth. The middle chamber is filled with physiological medium and is at extracellular potential. The large chambers on either end are filled with potassium chloride (120 mmol l<sup>-1</sup>) and are near intracellular potential. These three fluid compartments are isolated from each other by two narrow chambers, in which a flowing 'boundary' of sucrose electrically isolates the ends of the cord from its center (Fig. 4A). Stimulation at one end of the strip of cord produces compound action potentials (CAPs) that are conducted across the length of cord, and recorded at the other end. Stimulation and recording were carried out by conventional bridge circuitry, digitization, and data files were saved to computer files using a modified Labview program.

#### Statistical methods

MTT assay results are given as the mean  $\pm$  s.d. (standard deviation). Student's *t*-test was used to determine the significance between control, the group treated with acrolein, and the group treated with both acrolein and PEG. A *P* value <0.05 was considered significant.

#### Results

#### Cell death in populations of PC12 cells

We had established that a profound destruction of PC12 cells by necrosis occurred after exposure to 100 µmol l<sup>-1</sup> acrolein (Liu-Snyder et al., 2006a). In this study, application of 10 mmol 1<sup>-1</sup> PEG was unable to benefit these cells, as destruction of the population at 4 h after acrolein exposure was statistically similar to that in acrolein-treated cultures without the addition of the polymer. Fig. 1 shows the population responses to acrolein application in three groups: (1) a control group, in which PBS instead of acrolein was administered to the culture, (2) an acrolein-exposed group, and (3) an acroleinexposed group that was treated immediately after application of the toxin with PEG. Comparison of these photomicrographs shows that after 4 h, control PC12 cells were healthy and forming neurites (Fig. 1A'). There was no evidence in control cultures of cell debris or of rounded, unattached cells (an early indicator of necrosis). In stark contrast, the majority of the population of acrolein-treated PC12 cells were destroyed (Fig. 1B'). At the end of the 4 h trial period it was apparent that most of the population had died. What remained, other than the debris littering the substrate, was a small number of cells that had not died but were rounded, unattached and floating, and soon to die. This result was unaffected by the application of 10 mmol l<sup>-1</sup> PEG made within 15 min of exposure to acrolein (Fig. 1C').

#### A functional indicator of acrolein toxicity

MTT was used to further evaluate cell death in PC12 cells exposed to acrolein, since aberrant oxidative metabolism is indicative of acrolein-mediated cell death. Normally MTT is taken up into cells and reduced by a mitochondrial dehydrogenase to form a purple formazan product impermeable to cell membranes. Solubilization of the cells causes the release of the formazan product, which can be detected spectrophotometrically. The capability of cells to reduce MTT serves as an indicator of the mitochondrial activity, which is useful as a measure of viability. Fig. 2 shows the absorbances of experimental cultures. The formation of formazan product in PC12 cells was unaffected by application of PEG at the concentration and molecular mass used in these

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Fig. 1. Cell death after polyethylene glycol (PEG) application to acrolein-treated PC12 cells. (A–C) Control PC12 cells are shown developing on the culture substrate. Notice that these cells were well attached and some have extended processes giving them a stellate appearance. (A') The same culture as in A, 4 h after a 'sham treatment' – the introduction of PBS into the culture medium. Notice that all cells in this field of view are healthy, developing and all together similar to the culture 4 h previously. (B') The same culture as in B, 4 h after the introduction of 100  $\mu$ mol l<sup>-1</sup> acrolein. Note the significant loss of cells with cell debris littering the substrate. (C') The same culture as in C, showing significant cell death as a result of acrolein treatment, in spite of the addition of 10 mmol l<sup>-1</sup> PEG to the culture medium within 15 min after acrolein.

studies. A collapse in mitochondrial functioning, as revealed by the MTT assay, was typical of cells exposed to acrolein. Absorbance was reduced by over 95% by application of the toxin, when measured 4 h after exposure (Fig. 2). Finally, application of PEG was unable to remedy this collapse as the results of the MTT assay remained unchanged by an immediate application of PEG (Fig. 2).



Fig. 2. Acrolein, mitochondrial functioning and polyethylene glycol (PEG). Absorbance (ordinate) is an index of mitochondrial function/oxidative metabolism. Note that control cells (untreated) showed characteristic MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) absorbance spectra and the addition of PEG to the medium did not affect the MTT assay. Mitochondrial function, however, is markedly compromised by the addition of 100  $\mu$ mol l<sup>-1</sup> acrolein, and this result is not affected by the inclusion of PEG in the assay medium. \*Statistically significantly different from the control (*P*≤0.05).

### PEG attached to the PC12 outer cell membranes, but did not enter the cytoplasm

Evaluation of individual cells exposed to fluorescently decorated PEG revealed that within the first hour PEG was attached to the membrane surfaces of cells but did not enter the cytoplasm (Fig. 3). This result is difficult to directly observe in the two-dimensional images, shown in Fig. 3, of three-dimensional (3D) reconstructions of these cells. When the 3D reconstructions are 'animated' (i.e. the relative position of the cell to the observer is moved), however, it is much more apparent that the fluorescence is associated with the surfaces and not the interior of the cell being studied.

# Tissue level investigation: spinal cord white matter is vulnerable to acrolein

Guinea pig spinal cord ventral white matter was very resilient to oxygen and glucose deprivation (OGD) (see also Peasley and Shi, 2002; Peasley and Shi, 2003) (Fig. 4B,C). Strips of ventral white matter in the double sucrose gaprecording chamber recovered almost all of their initial propagation of CAPs after OGD for 60 min and reperfusing with normal oxygenated Krebs' solution for an additional 120 min (97.6±7%, N=8) (Fig. 4B,C). In another set of experiments, following a similar regimen of 60 min OGD, the strip was reperfused with 200 µmol l<sup>-1</sup> acrolein for 60 min, followed by another 60 min of reperfusion with normal Krebs' solution. The recovered CAP under these conditions was only 46.1±5% of the magnitude of the initial CAP recorded prior to OGD (N=6) (Fig. 5). Finally, in a 'rescue experiment', a similar experimental protocol was followed; however, PEG (10 mmol l<sup>-1</sup>; molecular mass, 2000 Da) was introduced into



Fig. 3. Polyethylene glycol (PEG) accumulates on the outside of PC12 cells. (A) 1 h after the addition of fluorescently decorated PEG, 3D confocal microscopy revealed that the only labeling above the limit of detection was confined to the surface of acrolein-treated cells. (B) By 2 h post treatment this same result was obtained, whereas at 3 h incubation time (data not shown), PEG was beginning to become apparent within the cells. These 2D representations, captured from 3D animated data sets, do not easily reveal the location of fluorescence when confined to the surface. Animating the 3D reconstruction, however, confirmed this surface labeling.

the perfusion in addition to the acrolein (60 min). The CAPs following 120 min of reperfusion were  $45.7\pm4.6\%$  (*N*=6) of the initial CAPs recorded before the ischemic insult. This result was not significantly higher than that of the ischemia–acrolein group (Fig. 5; *P*>0.05). In a separate control experiment, uninjured control cords were exposed to PEG for 60 min and no significant change in amplitude of CAPs resulted (*N*=5, *P*>0.05).

#### Discussion

Acute PEG application was unable to protect relatively intact cells and axons from necrotic death in response to the products of LPO. After a 4 h exposure to 100  $\mu$ mol l<sup>-1</sup> acrolein, a nearly

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complete destruction of cultured PC12 cells occurred. We also detected this toxicity using MTT neurochemistry, an indirect indicator of oxygen metabolism in cells. Furthermore, this cell death was not affected by application of 10 mmol l<sup>-1</sup>, 2000 Da PEG to the culture. In guinea pig spinal cord tissue maintained in a recording/isolation chamber, we also show that the rapid collapse of nerve impulse conduction associated with oxygen and glucose deprivation and acrolein exposure is likewise not rescued by PEG application. The molecular mass and concentration of PEG was typical of that used in prior studies where injured brain tissue, and spinal cord in vivo and in situ, was clearly protected by polymer administration (Koob et al., 2005; Shi and Borgens, 1999; Borgens and Shi, 2000; Laverty et al., 2004). The range of molecular mass used in these studies was based on the literature of PEG-mediated cell fusion and an evolution of practical concerns about administering the polymer in laboratory animal and canine clinical studies (~1000-3000 Da) (Borgens and Shi, 2000; Davidson et al., 1976; Laverty et al., 2004; Borgens, 2001).

Furthermore, confocal microscopy of the interaction of PEG with acrolein-poisoned cell membranes supports the conclusion that PEG was unable to enter the cytoplasm of poisoned cells before 2 h, which was too little time to provide neuroprotection against the progressive toxicity sweeping the culture or spinal cord tissues. These results further suggest that PEG must enter the cytosol to be an effective 'antidote' to the endogenous toxicity associated with secondary injury in relatively intact cells in or near a region of mechanical damage to soft tissues.

#### Secondary injury

The term itself has an interesting and lengthy history. In the modern era of neurotrauma, the worsening of the lesion with time after injury is a factual observation. The mechanisms of this worsening became of interest in the late 1970s, coincident with the understanding of biochemically mediated cell death; necrosis and apoptosis. Initially pioneer studies of secondary injury focused on the role of reactive oxygen species (so-called free radicals) as the mediators of cell death in mechanically injured cells. Though the relationship between reactive oxygen species and lipid peroxidation was emerging together, it is interesting to point out that the experimental use, and later clinical adoption, of methylprednisilone sodium succinate as a therapeutic agent in SCI was driven by the emerging story of free radicals and the possibility of combating their deleterious effects with various antioxidant strategies. In neurotrauma the use of steroids, particularly glucocorticoids, was not based, for example, on their ability to reduce swelling. Moreover, these agents were curiously contraindicated as interventions in ischemic head injury and stroke at about this same time (Sapolsky and Pulsinelli, 1985). The results of animal experimentation in neurotrauma based on the reduction or nulling of ROS has proved to be mixed, whereas human clinical experimentation using antioxidant 'therapy' has failed. It is reasonable to suggest that emphasis on ROS was unbalanced given the extraordinary amounts and lethality of toxins produced, which were only partially the result of reactions

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Fig. 4. The effects of polyethylene glycol (PEG) on CAP recovery following ischemia/acrolein reperfusion. (A) The double-sucrose gaprecording chamber. The device is constructed of Plexiglas<sup>TM</sup> with five separate chambers, all linked together by a narrow slot. Three large chambers are shown; the two end ones filled with KCl and the middle chamber with a physiological medium (a modified Krebs' solution), where a constant flow of medium through the chamber is carried out. The delivery to an 'antechamber' reduces any turbulence or artifacts based on flow of medium through and out of the device. A delivery and an aspiration tube (that sets the fluid level) are shown for the central chamber and one of the small sucrose chambers to its right. A constant flow of sucrose significantly reduces the mixing of the KCl solution in the end chambers with the center one. A full length (~40 mm) of guinea pig spinal cord or a wedge-shaped long strip of only ventral white matter was placed across and within all five chambers. The ends of the dissected cord are then near intracellular potential, while the center of the cord is near extracellular potential. Bipolar stimulating electrodes fire compound action potentials (CAPs) at one end of the cord, and these are recorded arriving at the other end with bipolar recording electrodes. This arrangement provides a very precise recording of CAPs in cord for many hours at a time. It also allows the addition of test drugs or other interventions to be carried out in the central chamber. (B) The CAP amplitude profile in (a) the presence of oxygen and glucose deprivation (OGD), (b) OGD plus acrolein, and (c) OGD plus acrolein plus PEG. This graph displays the CAP amplitude recorded over a period of time. The values were normalized. Note the similarity in CAP amplitude recovery during the reperfusion period for groups (b) and (c). (C) The CAP waveforms are shown at three time points, (a) pre OGD, (b) at the end of OGD and (c) at the end of reperfusion, as indicated in B. Note there is little difference from the initial CAPs and the ones recorded following recovery from ischemia in OGD group. However, the amplitude is reduced to a half of pre-OGD levels in both groups (b) and (c). Scale bars, 1 mV, 1 ms.



Fig. 5. Compound action potential (CAP) recovery in oxygen and glucose deprivation (OGD), OGD plus acrolein, and OGD plus acrolein plus polyethylene glycol (PEG) groups. In the OGD alone group, there was a 97.6 $\pm$ 7% recovery of the CAP following an ischemic insult (*N*=8). The CAP recoveries of the OGD plus acrolein and the OGD plus acrolein plus PEG groups were 46 $\pm$ 5% and 45.7 $\pm$ 4.6%, respectively. *N*=6 in each group. \**P*=0.88; Student's *t*-test, unpaired, two tail.

catalyzed by these short-lived intermediate species. The halflives of ROS such as superoxide in physiological conditions are only in the order of picoseconds, whereas toxins like acrolein and HNE are true poisons and can remain in tissues and body fluids for many hours to days, crossing intact cell membranes, stimulating their own production, and killing nearby cells.

#### Polymer-mediated rescue from 'secondary injury'?

The neuroprotective capability of PEG and the poloxamers is based on their action in both fusing (Shi et al., 1999) and sealing (Shi and Borgens, 1999; Shi and Borgens, 2000) neurons and their processes. In model membranes, it has been determined that this action is characteristic of polymers and surfactants that are amphiphilic and hydrophilic. It is a polymer-induced alteration of the organization of water at the surface of cells that first allows an intermingling of the outer leaflets of the membrane, proceeding on to membrane fusion and then cell fusion (see Nakajima and Ikada, 1994). In mechanically injured membranes, this strong hydrophilia reduces the aqueous phase, which is inserted deep into - and between - the 'broken' lipidic phases of the membrane. Reduction in this aqueous phase permits the portions of the membrane's core to resolve into each other. Since many structural proteins and receptors are architecturally dependent on polar forces associated with the membrane, its reconstitution furthers allows a form of spontaneous self-assembly (reviewed by Borgens, 2001; Borgens, 2003).

Our interest in understanding the beneficial action of PEG and P188 (Borgens and Shi, 2000; Borgens and Bohnert, 2001; Borgens et al., 2002; Laverty et al., 2004) in experimental neurotrauma has also required us to determine if PEG could act as a 'free radical scavenger' as has been suggested for poloxamers (Marks et al., 2001).

Whereas PEG application indeed reduced the concentration of ROS in damaged spinal cord (Luo et al., 2002), it did not act as an antioxidant in neurochemical tests of this capability.

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For instance, PEG was not able to inhibit xanthine xanthine oxidase catalysis of free radical production, or to act similarly as known potent anti-oxidants such as ascorbic acid, SOD and allopurinol in cell-free extract studies (Luo et al., 2002). This result has led us to postulate that PEG may reduce the concentration of free radicals by a direct action in repairing the membrane of mitochondria and so reduce the aberrant oxygen metabolism that is a result of their destabilization (Luo et al., 2004). To do this PEG must enter the cytosol. This has been directly observed in mechanically damaged cells by using a fluorescently decorated PEG (Luo et al., 2004).

The issue of a possible action of PEG in reducing the severity of secondary injury seemed likely to depend on PEG entering cells that have relatively intact cell membranes, but are compromised. Such destruction is characteristic of SCI, and is often referred to as 'bystander damage'. This occurs in normal and undamaged cells in the vicinity of deteriorating soft tissue by the creation of a toxic environment spreading out from the epicenter of the lesion. For example the high levels of K<sup>+</sup> that exists in the extracellular fluid of CNS lesions, and the increasing concentrations of toxins produced locally by the collapse and death of injured cells create this environment. The aggregation of macrophages also contributes to this *via* the catabolic products they produce during active phagocytosis, including their ability to partially demylenate axons creating conduction block.

The results of this study emphasize that (1) polymer application may not be able to interfere with these progressive problems in cells with relatively intact membranes (which appear to inhibit polymer movement into the intracellular compartment), and (2) the final and catastrophic outcome from acute mechanical damage to the CNS is due to processes that are extraordinarily complex and will likely require several 'therapies' acting in concert to produce interventions that are clinically useful.

#### List of abbreviations

CAP	compound action potential
DMEM	Dulbecco modified Eagle's medium
HNE	4-hydroxynonenal
HRP	horseradish peroxidase
LDH	lactic dehydrogenase
LPO	lipid peroxidation
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl
	tetrazolium bromide
OGD	oxygen and glucose deprivation
PBS	phosphate-buffered saline
PEG	polyethylene glycol
ROS	reactive oxygen species
SCI	spinal cord injury
TBI	traumatic brain injury

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