

Statistical summary

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Non-invasive topical drug delivery to spinal cord with carboxyl-modified trifunctional copolymer of ethylene oxide and propylene oxide

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A B S T R A C T

In this study the effect of oxidative modification on micellar and drug delivery properties of copolymers of ethylene oxide (EO) and propylene oxide (PO) was investigated. Carboxylated trifunctional copolymers were synthesized in the reaction with chromium oxide (VI). We found that carboxylation significantly improved the uniformity and stability of polymeric micelles by inhibiting the microphase transition. The cytotoxicity of copolymers was studied in relation to their aggregative state on two cell types (cancer line vs. primary fibroblasts). The accumulation of rhodamine 123 in neuroblastoma SH-SY5Y cells was dramatically increased in the presence of the oxidized block copolymer with the number of PO and EO units of 83.5 and 24.2, respectively. The copolymer was also tested as an enhancer for topical drug delivery to the spinal cord when applied subdurally. The oxidized copolymer facilitated the penetration of rhodamine 123 across spinal cord tissues and increased its intraspinal accumulation. These results show the potential of using oxidized EO/PO based polymers for non-invasive delivery of protective drugs after spinal cord injury.

Keywords: Amphiphilic polymers; Trifunctional copolymers of ethylene oxide and propylene oxide; Oxidative modification; Micelles; Drug delivery; Neural cells; Spinal cord; Traumatic injury

1. Introduction

Although a number of synthetic and biological substances were suggested as neuroprotectors, there is still a lack of available therapy for neurotrauma and particularly for spinal cord (SC) injury [1]. The only clinically accepted substance to cure the acute spinal cord injury is high-dose intravenous infusion of methylprednisolone, which is nevertheless controversial and related with serious side effects [2].

A line of potential agents with the neuroprotective activity has been studied over the last years, such as antioxidants [3], antagonists for glutamate excitotoxicity [4], and others. Recently polymers and colloids attract interest of researchers as promising neuroprotective agents and also a system for drug delivery.

Borgens et al. demonstrated that the administration of low molecular weight PEG to guinea pigs after severe spinal cord injury results in a significant recovery of nerve conduction and inhibition of consequences of secondary trauma [5,6]. Several mechanisms for PEG-assisted neuroprotection have been proposed, including direct sealing of the plasma membrane of damaged nerve cells, inhibition of calcium influx and oxidative stress, mitochondria protection and apoptosis inhibition [5,6]. The polymer-based therapy has been extended to PEG-related amphiphilic polymers, e.g. the block copolymer of ethylene oxide (EO) and propylene oxide (PO) Pluronic F68 (Poloxamer 188) [7] and PEG–polylactic acid (PLA) copolymer [8], which exhibited neuroprotective effect in SC injury at lower dose than that for PEG.

Other approaches to treating SC injury have been focused on the local polymer-assisted transfer of bioactive substances to the damaged spinal cord. Most studies in this field have reported gel and nanoparticle-based formulations with encapsulated drugs, which are intrathecally injected in the SC. Gupta et al. proposed fast-gelling hyaluronan/methylcellulose composite which exhibited biocompatible properties upon prolonged implantation in the SC [9]. Kim et al. developed methylprednisolone-loaded poly(lactic-co-glycolic acid) nanoparticles prepared by the double emulsion method. The local injection of nanoparticles in the SC helps to prolong drug delivery in a more effective compared to systemic administration way [10].

Furthermore, photoactivated biodegradable hydrogel synthesized from acrylated PLA–PEG–PLA block copolymer has been proposed by Piantino et al. as an injectable formulation releasing the trophic factor neurotrophin-3 to promote the axonal growth and functional recovery of damaged spinal nerves [11]. Recently, polyacrylate/agarose composite hydrogels, containing poly(methyl methacrylate) nanoparticles, with different porosity were developed for the sustained and controllable release of both small-molecule drugs and nanoparticles. Drug-releasing properties of these hydrogels were also studied after their intrathecal injection in mouse spinal cord [12].

Compared with invasive injections of polymeric systems, the topical drug delivery across SC membranes is expected to be safer, however it requires appropriate enhancers for drugs transport to the site of injury. Except of relatively toxic dimethyl sulfoxide vehicle [13], to this time no safe and effective enhancers have been proposed for drug delivery to the SC.

Micelle-forming non-ionic amphiphilic polymers are one of the most promising delivery systems for anticancer and CNS-directed drugs. Among them, systematically studied bifunctional $(EO)_x-(PO)_y-(EO)_x$ block copolymers, Pluronic®, have been shown to modulate functional activity of cellular membranes and increase their permeability to different drugs [14]. Other amphiphilic polymers, including non-ionic PEG–PLA [15], charged quaternised poly(propylene imine) dendrimer [16] and thioglycolic acid-modified methoxy PEG-b-poly(5-allyloxy-1,3-dioxan-2-one) [17] were recently proposed as micellar carriers for anticancer and anti-inflammatory compounds.

We demonstrated previously that the introduction of anionic groups into EO/PO copolymers [18] and the use of their tri-functional glycerol-based analogs, exhibiting structural similarity to membrane lipids [19], are promising approaches to developing safe and potent polymeric carriers and enhancers. In this study, chemically oxidized trifunctional copolymers of EO and PO with improved micellar properties were synthesized. Our findings demonstrate for the first time that the oxidized copolymers can enhance topical drug delivery to the spinal cord.

2. Experimental

2.1. Reagents and materials

Trifunctional copolymers of PO and EO (Laprol® 5003, Laprol® 6003, structural analogs of Voranols, Dow Chemical) were produced by JSC Nizhnekamskneftekhim (Russia). Pluronic block copolymers of PO and EO (Pluronic® L61, Pluronic® F68) were produced by Sigma-Aldrich.

Polyacrylic acid (Carbopol 934) was produced by Serva. Chromium oxide (VI), *p*-formaldehyde, glutaraldehyde (25 % solution), pyrene, chloral hydrate, organic solvents and inorganic salts were purchased from Acros Organics. Rhodamine 123, 4',6-diamidino-2-phenylindole (DAPI) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma-Aldrich. Neg-50 frozen section medium was purchased from Thermo Scientific.

Cell culture reagents were purchased from PAA Laboratories.

2.2. Synthesis and characterization of oxidized copolymers

Copolymers of EO and PO were oxidized by CrO₃ according to previously proposed method for PEGs [20]. Briefly, the copolymer (5–15 g) was mixed with CrO₃ at a ratio of 1 : 3 by mole in 25 % solution of sulfuric acid. The mixture was incubated for 24 hrs under constant stirring. The product was extracted with a double volume of dichloromethane (×3), and washed with 1/10 volume of water (×2) and 1/10 volume of saturated sodium chloride solution (×1). The extract was dehydrated under anhydrous magnesium sulfate and dried on a rotary evaporator. The products were oily substances with yellowish color. The yield was 65 and 67 % for modified Laprol 5003 and Laprol 6003, respectively.

The presence of carboxyl groups in modified copolymers were verified by a FTIR spectroscopy on a Frontier spectrometer (Perkin Elmer) using the following parameters: wavenumber region 4000–400 cm⁻¹, resolution 1 cm⁻¹, scan number 64. The modification extent was determined by means of acid-base titration with 0.01 M potassium hydroxide as a titrant and Nile blue as a pH indicator. Complete conversion of three terminal

hydroxyls in trifunctional copolymers into carboxyl groups was assumed equal to 100 % modification.

2.3. High-performance liquid chromatography

Chromatographic analysis of integrity of oxidized copolymers was carried out on an Ultimate 3000 HPLC system (Dionex / Thermo Fisher Scientific). Copolymers dissolved in HPLC-grade methanol were separated on a Zorbax SB-C18 column with dimensions 4.6×150 mm and pore size of 80 Å (Agilent). The isocratic mode was used with the following parameters: methanol / water (40 / 60) mobile phase; flow rate 0.5 mL/min; UV-detector 220 nm. Chromatographic data acquisition and processing was performed with the aid of Chromeleon 6.80 software.

2.4. Study of micelle-forming properties of oxidized copolymers

The critical micellar concentration (CMC) of copolymers was determined with the use of pyrene probe in PBS at room temperature as described in [18]. Copolymer aggregates were further analyzed by the dynamic light scattering (DLS) technique on a Zetasizer Nano ZS instrument (Malvern Instruments). Hydrodynamic diameter and zeta potential were registered in 50 mM HEPES buffer (pH 7.0) or in DMEM with 5 % fetal bovine serum at copolymer concentrations of 0.2 and 2 mg/mL, respectively. The measurements were performed in triplicates.

2.5. Cell culturing

Primary human skin fibroblasts (HSFs) were isolated from skin explants according to a conventional procedure [21]. Human neuroblastoma SH-SY5Y cells were obtained from ATCC. HSFs were cultured in α -MEM supplemented with 10 % FBS, 2 mM L-glutamine, 100 μ g/mL streptomycin and 100 U/mL penicillin under standard conditions (37 °C, humidified atmosphere of 5 % CO₂). SH-SY5Y cells were grown in the same conditions, but in DMEM medium.

2.6. MTT-assay

Cytotoxic concentrations (IC_{50}) of copolymers were determined with the use of MTT assay. Cells were pre-seeded in 96-well plate at the density of 1000 cells per well and cultured with adding serially diluted copolymer solutions under standard conditions. After 3 day culturing, the medium was replaced by the fresh one supplemented with 0.5 mg/mL MTT reagent, which was further kept for 4 hrs to reduce MTT into colored formazan by viable cells. Optical absorption of formazan, proportional to the number of viable cells, was registered on Infinite M200 PRO analyzer (Tecan) at the wavelength of 550 nm.

2.7. Study of rhodamine 123 accumulation in SH-SY5Y cells

SH-SY5Y cells were collected and suspended in PBS at a density of 1.5×10^6 cells/mL. An aliquot of the copolymer solution was added to the cell suspension in 1.5 mL polystyrene tubes to obtain final concentration of 1 mg/mL. After 15 min pre-incubation with a copolymer, the cell suspension was supplemented with 5 μ M rhodamine 123 and additionally kept for 30 min at 37 °C under mild agitation. The treated cells were washed twice with chilled PBS by means of centrifugation and lysed in 1 mg/mL Triton X100 in deionized water solution. Cell lysates were then transferred into 96-well plates, followed by the detection of rhodamine 123 fluorescence on Infinite M200 PRO analyzer at λ_{ex} 480 nm and λ_{em} 530 nm.

2.8. Preparation of topical gel formulation

Polyacrylic acid was solubilized and neutralized with sodium hydroxide to obtain 4 wt% viscous gel (pH 7.0). The gel was further mixed with equally amount of 1 wt% copolymer solution in PBS containing 100 μ M rhodamine 123 to produce the topical gel formulation containing 2 wt% polyacrylate, 0.5 wt% L6-COOH as a penetration enhancer and 50 μ M rhodamine 123 as a delivered compound.

2.9. Topical delivery of rhodamine 123 to rat spinal cord

Eight adult male Wistar rats (250–400 g) were used for in vivo study. Animal care was performed according to European regulations on the protection of experimental animals (Directive 2010/63/UE) and current Russian regulations (No.742 from 13.11.1984, (Ministry of Education and Science). The study was approved by the Ethic committee of Kazan Federal University (Russia).

All surgical procedures were carried out under aseptic conditions. Rats were anesthetized deeply using isoflurane inhalation. Laminectomy was performed at Th13 vertebral segment and the dura mater was cut to expose the spinal cord (SC). Both control and experimental groups included four animals. Polyacrylate gels loaded with rhodamine 123 (control group) or rhodamine 123/copolymer mixture (experimental group) were accurately applied onto the arachnoid membrane of SC using insulin syringe (100 µL gel aliquot per segment) and kept for 2 hrs. After exposure the gel was washed out with PBS. Animals were additionally narcotized by the intraperitoneal injection of chloral hydrate at a dose of 300 mg/kg and subjected to a conventional transcardial perfusion with 4 % formalin in PBS (500 mL per animal). Treated SC segments of ~1 cm in length were dissected and immediately fixed with 4 % p-formaldehyde in PBS solution containing 0.1 % glutaraldehyde for 3 hrs, followed by washing with PBS and freezing in liquid nitrogen for the sectioning.

2.10. Laser scanning confocal microscopy of spinal cord sections

20 µm thick cross-sections of pre-fixed SC were prepared using a Microm HM 560 cryostat (Thermo Scientific) and mounted onto pre-modified glass slide for laser scanning confocal microscopy (LSCM). For cell nuclei staining, glass-adhered SC sections were incubated with 0.3 µM DAPI in PBS for 5 min, rinsed with milli-Q water and dried. The samples were visualized on an Axio Observer. A1 fluorescent microscope and a confocal microscope Zeiss LSM 780 (Carl Zeiss) using the argon laser and 5× air objective.

The quantity of rhodamine 123 delivered into the SC was assessed from LSCM images using ImageJ software (US National Institutes of Health). SC sections were divided into

4 equal quarters, and the intensity of rhodamine 123 fluorescence was quantified by measuring the integrated density parameter of the dorsal quarter normalized by its area in μm^2 . At least six LSCM images per group were analyzed to estimate the effect of oxidized copolymer on rhodamine 123 penetration into the spinal cord.

Data are reported as mean \pm SEM. The statistical significance between control and experimental groups was verified by the Wilcoxon test. The depth-dependent distribution of rhodamine 123 in SC sections was analyzed by measuring the fluorescence along diffusion direction starting from the gel-contacting site to the spinal canal with a step of 100 μm . The criterion level for determination of statistical significance was set as $p < 0.05$ for all comparisons.

3. Results and discussion

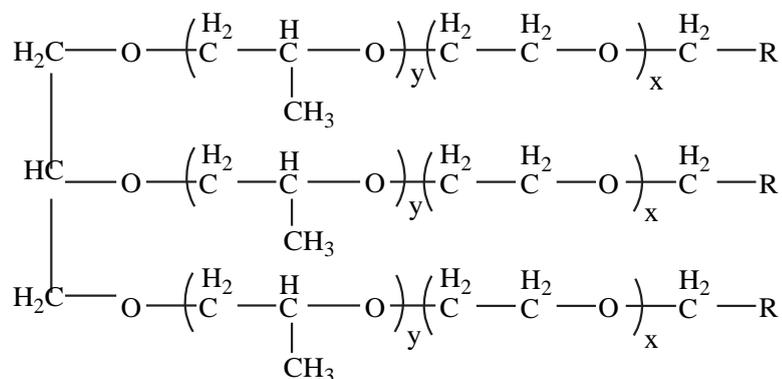
3.1. Characterization of oxidized copolymers

Two glycerol-based trifunctional copolymers of EO and PO, a random copolymer Laprol 5003 (L5) and a block copolymer Laprol 6003 (L6) were selected as bifunctional pluronic analogs with enhanced ability to modulate cellular membranes [19,22]. Along with the primary structure, L5 and L6 copolymers differ in their number average molecular weight (MW), EO/PO ratio and hydrophilic lipophilic balance, HLB (Table 1).

We showed previously that the introduction of anionic succinate moiety into different EO/PO copolymers in the reaction with succinic anhydride increased cellular compatibility and delivery properties of polymeric molecules [18,19]. In this study, terminal hydroxyl groups in trifunctional copolymers were directly converted into carboxyl groups by means of direct oxidation, and the effect of this modification was examined.

The copolymers were oxidized in the reaction with CrO_3 as described in the Experimental section. FTIR spectra of CrO_3 -oxidized copolymers contain a specific band at 1735 cm^{-1} wavenumber (Supplementary data, Fig. 1S) which corresponds to the carboxyl group formed during the oxidation of terminal OH. According to the acid-base titration, the OH conversion rate was almost 75 % for the oxidized L5 (L5-COOH) and

80 % for the oxidized L6 (L6–COOH), which corresponded to 2.3-2.4 modified groups per the trifunctional copolymer molecule. The general chemical structure of modified trifunctional copolymers is provided as follows, where x and y is the number of EO and PO units, respectively; and R: –CH₂OH or –C(O)OH.



Additionally, Pluronic L61 (MW 2000, HLB 3) and Pluronic F68 (MW 8400, HLB 29) were reacted with CrO₃ to produce oxidized polymers with 67 and 68 % modification rate, respectively. The results show that CrO₃ can be used to oxidize both random and block EO/PO based copolymers which differ in their HLB, MW and functionality.

A reverse-phase HPLC analysis of the integrity of oxidized copolymers showed no change in the retention time for initial and modified copolymers (Supplementary data, Fig. 2S). This indicates that the oxidative modification by CrO₃ does not result in the destruction of copolymer molecules. Together with a satisfactory yield, this makes the proposed approach useful for the synthesis of carboxyl-modified copolymers of EO and PO. In contrast to the reaction with succinic anhydride, which produces hydrolysable succinic acid ester [18,19], the oxidative modification should provide more stable functionalization of copolymers as the introduced carboxyl group is not expected to undergo hydrolysis.

3.2. *Micelle-forming properties of oxidized copolymers*

The critical micellar concentration (CMC) of initial and oxidized copolymers was determined with the use of pyrene probe. L6 was found to have lower CMC value (50.9±11.8 µg/mL) in comparison with L5 (111.2±14.7 µg/mL), reflecting a relatively

better ability of the former copolymer to aggregate into micelles apparently due its higher molecular weight. Modified copolymers were characterized by statistically non-significant change in CMC values compared with unmodified counterparts (63.3 ± 12 $\mu\text{g/mL}$ for L6-COOH and 98.0 ± 7.7 $\mu\text{g/mL}$ for L5-COOH). This suggests little or no effect of the oxidative modification of copolymers on their micelle formation.

As reported earlier, the succinimidation of L6 and Pluronics was accompanied by a noticeable CMC decrease for the modified copolymers [18,19]. The results indicate that the esterification with succinic acid promotes copolymers aggregation in water solution apparently due to the increase in their MW and/or decrease in HLB, while the conversion of terminal OH into COOH does not significantly alter physicochemical characteristics of copolymers.

Copolymer aggregates were further analyzed with the use of DLS technique in HEPES buffer (25 °C), where both hydrodynamic diameter and zeta potential of micelles were measured, and in fetal bovine serum (FBS) containing DMEM medium (37 °C) to model the cell culture environment. In HEPES buffer L5 and L6 copolymers appear as large micelles which tend to form micro-sized aggregates (microphase) due to their relatively low HLB values (Fig. 1A).

In buffer solution oxidized copolymers were characterized by a smaller diameter and lower particle dispersion index (PDI) compared with unmodified ones. The average size of polymeric aggregates decreased in the range: L5 (330.2 ± 4.1 nm, PDI 0.291); L6 (303.3 ± 7.1 nm, PDI 0.322); L5-COOH (119.1 ± 0.7 nm, PDI 0.221); L6-COOH (96.4 ± 0.9 nm, PDI 0.155), where L6-COOH copolymer formed the micellar system with lowest size and dispersity (Fig. 1A).

The electrokinetic analysis showed that initial non-ionic copolymers have slightly negative zeta potential of -8.1 ± 1.1 mV for L5 and -6.1 ± 0.3 mV for L6 attributed to the presence of electronegative hydroxyl groups on the surface of polymeric micelles. The oxidative modification of L5 and L6 copolymers resulted in a significant increase in their zeta potential up to -31.2 ± 4.0 mV for L5-COOH and -24.2 ± 4.0 mV for L6-COOH due to the appearance of anionic carboxyl function.

In 'cell culture' conditions copolymer concentration was raised to 2 mg/mL due to the interference from medium components. Although increased temperature (37 °C) should

promote the hydrophobic interactions of EO/PO based copolymers as a result of their dehydration [23], no significant increase in the diameter of copolymer aggregates was observed in these conditions (Fig. 1B), presumably due to a protective effect of serum proteins [24].

In DMEM/FBS modified copolymers L5-COOH and L6-COOH also formed smaller micelles with low PDI values near to 0.1 (Fig. 1B), which is characteristic for a mono-dispersed system [25]. The results demonstrate that the oxidized copolymers generate more stable and uniform micelles compared with unmodified counterparts, which tend to form micro-sized aggregates. Therefore, the introduction of anionic carboxyl group into non-ionic copolymers does not inhibit their micelle formation but prevents further micelle aggregation to the microphase. This effect is of particular interest for improving micellar and pharmacokinetic properties of amphiphilic copolymers of EO and PO as drug carriers and enhancers [24,26].

3.3. Effect of oxidized copolymers on cell viability and rhodamine 123 uptake

A comparative study of cytotoxic activity of initial and modified copolymers was carried out with the aid of proliferative MTT-assay. Table 2 summarizes cytotoxic concentrations (IC_{50}) of copolymers for primary human skin fibroblasts (HSFs) and neuroblastoma SH-SY5Y line used as a neuron-like model [27].

Unmodified L5 and L6 exhibited IC_{50} values for HSFs of about 0.1 and 1.4 mg/mL, respectively, indicating a moderate cellular toxicity in experimental conditions. Cytotoxicity of these copolymers was found to be significantly higher for SH-SY5Y cells with IC_{50} values of almost 6 and 24 μ g/mL, respectively (Table 2). This is in accordance with our previous results which showed that EO/PO copolymers preferably induce death in cancer cells than primary fibroblasts. The observed selectivity suggests ‘intracellular mode’ of cytotoxicity of amphiphilic copolymers as cancer cells have more intense metabolism and higher proliferative potential, and therefore more readily accumulate copolymers in their interior [19,22]. Inside cells EO/PO copolymers were shown to interact with subcellular structures, including mitochondria, impairing some metabolic processes [28].

Oxidative modification of copolymers resulted in an apparent decrease of their IC_{50} values (Table 2). The increase in cytotoxicity observed for modified copolymers could be explained from their micelle-forming properties. As shown above, initial copolymers readily turn to the microphase in the culture medium (Fig. 1B), which should result in a significant decrease in their real concentration affecting cell viability. Modification of copolymers reduces their aggregation (Figs. 1A and 1B) and increases their effective concentration in micellar and unimer forms, which are capable of interacting with mammalian cells [14,23].

Further analysis of dose-response curves showed that oxidized copolymers exhibited somewhat higher cytotoxicity than unmodified ones only in higher concentration range above 0.1 mg/mL (Fig. 2), where significant difference in copolymer aggregation was observed (Fig. 1). This demonstrates an importance of the aggregation state of EO/PO based amphiphilic polymers for their biological activity.

Altogether, our results support that the oxidative modification of trifunctional copolymers does not induce an actual increase in their toxicity for human cells. However it is expected to increase their effective concentration and availability for biological interactions affecting membrane-modulating and drug delivery properties.

An ability of copolymers to promote intracellular drug delivery was assessed on SH-SY5Y cells using rhodamine 123 as a pharmacological model for cellular influx and efflux transport [19]. Cells were incubated with rhodamine 123 in the absence or in the presence of copolymers as a transport enhancer, and the accumulated fluorophore was determined in cell lysates. The cellular uptake of rhodamine 123 alone was found to be relatively low, but it was significantly improved by copolymers. The promoting effect of copolymers increased in the range: L5, L5-COOH, L6, L6-COOH, where L6-COOH copolymer enhanced the intracellular accumulation of rhodamine 123 with the highest factor of 8.2 (Fig. 3).

We believe that penetration enhancement of rhodamine 123 into SH-SY5Y cells by trifunctional copolymers could be explained by their structural similarity with membrane glycerolipids, which determines their high membranotropic and membrane-modulating activity. As shown earlier, such copolymers reversibly disturb the plasma membrane and increase its permeability to small molecules [19]. The introduction of carboxyl function

into EO/PO copolymers may enhance such an activity through different mechanisms [18,19], e.g. the potentiation of their effect on cellular membranes or/and the complex formation with a molecule transported. In contrast to tri-functional copolymers, no enhancing effect on rhodamine 123 transport was observed for initial and oxidized Pluronic L61, while Pluronic F68 somewhat inhibited cellular uptake of the fluorophore in the same conditions (data not shown). This indicates that above Pluronic do not increase permeability of the plasma membrane of SH-SY5Y cells presumably do to the lack in their ability to be inserted into the lipid bilayer. On the basis of above results, L6-COOH copolymer was chosen as a candidate for drug delivery in vivo. The oxidized copolymer was further examined as a drug delivery system to the spinal cord in a limited animal study.

3.4. Topical delivery of rhodamine 123 to the spinal cord

A rat model with exposed segments of the spinal cord after lamina bone and dura mater dissection was prepared as described previously [29,30]. Polyacrylate gels containing rhodamine 123 with and without L6-COOH copolymer were applied topically onto open Th13 segment for two hours. LSCM analysis of a series of cross sections of the SC segments after treatment with gel formulations was carried out.

We found that rhodamine 123 alone was delivered across the SC membranes, the arachnoid membrane and pia matter, and appeared in different zones of white and grey matter (Fig. 4A). The dye penetration into the spinal cord from the gel was noticeably promoted by L6-COOH copolymer, which resulted in the increase of fluorescence area in zones adjacent to the gel-contacting site (Fig. 4B).

The mean fluorescence of penetrated rhodamine 123 was estimated with the aid of ImageJ software by calculating the area-normalized integrated density in the dorsal quarter of SC sections in two groups. This parameter was almost 2.1-times higher in the experimental group, when L6-COOH copolymer was applied, compared with the control group (Fig. 5), indicating a noticeable promotion of the intraspinal delivery of rhodamine 123 by the oxidized copolymer.

Depth-dependent distribution of rhodamine 123 in SC sections was further assessed by measurement of fluorescence along the diffusion direction towards the spinal canal (Supplementary data, Fig. 3S). An exponential decrease of intraspinal penetration of rhodamine 123 was observed with distance; L6-COOH enhanced the penetration depth over 1200 μm , and its effect was somewhat more evident in deeper layers (Fig. 3S).

Our study demonstrates that the oxidized tri-functional copolymer of EO and PO (L6-COOH) promotes the delivery of model fluorophore to undamaged spinal cord tissues. Due to its relatively high molecular weight and micelle-forming properties, L6-COOH copolymer is not expected to rapidly diffuse across intact SC membranes. Therefore, the copolymer apparently affects the permeability of spinal cord coverings, leading to enhanced penetration of the probe. The glia limitans beneath the pia matter separating spinal cord tissues is one of the possible lipophilic barriers affected by the oxidized copolymer to increase the intraspinal delivery. The rupture of SC tissues upon traumatic injury could promote copolymer accumulation and also increase drug delivery at the cellular level, as shown for SH-SY5Y cells (Fig. 3).

Further experiments should be performed to study mechanisms of the promoting effect of L6-COOH copolymer and its therapeutic potential for treating SC injury. Altogether, our results contribute to the development of polymeric systems for advanced drug delivery into living cells and could be considered for topical treatment of acute spinal cord injury.

4. Conclusions

The lack of effective delivery systems for neuroprotective drugs remains a big challenge in treating acute neural injury. Tri-functional copolymers of ethylene oxide and propylene oxide appear to be a promising platform for the development of safe and effective drug carriers *in vivo*. Our results indicate that oxidative modification of these copolymers, which introduces the terminal carboxyl functions, significantly improves characteristics of polymeric micelles and promote polymer-mediated accumulation of fluorescent probe in neuron-like cells. For the first time we demonstrate that oxidized

copolymers facilitate topical delivery to the intact spinal cord tissues. The results of our study could potentially help to develop effective drug compositions for the treatment of traumatic neural injury.

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Table 1

Physicochemical characteristics of initial and oxidized trifunctional copolymers of EO and PO.

Copolymer	Number average MW (Da)	Number of PO units per molecule	Number of EO units per molecule	Modification degree (%)	HLB*
L5	5000	71.9	16.7	0	6.0
L5-COOH	~5000	71.9	-	75	-
L6	6000	83.5	24.2	0	6.7
L6-COOH	~6000	83.5	-	80	-

*HLB was calculated theoretically by the Davies method.

Table 2

Cytotoxic concentrations (IC₅₀, µg/mL) of initial and modified copolymers of EO and PO (MTT assay).

Copolymer	HSFs	SH-SY5Y cells
L5	112.7±35.2	6.4±0.6
L5-COOH	73.2±14.0	7.4±4.3
L6	1409.4±285.5	23.9±3.8
L6-COOH	393.0±38.0	15.8±8.2

Figure captions

Fig. 1. Distribution of hydrodynamic diameter of initial and oxidized copolymers of EO and PO: (A) 50 mM HEPES, pH 7.0, temperature 25 °C, copolymer concentration 0.2 mg/mL; (B) DMEM with 5 % FBS, temperature 37 °C, copolymer concentration 2 mg/mL.

Fig. 2. Dose-response curves for HSFs cultured in the presence of copolymers: (A) L5, L5-COOH; (B) L6, L6-COOH (MTT-assay). (□) Initial copolymers, (○) oxidized copolymers.

Fig. 3. Fluorescent signal of rhodamine 123 accumulated in SH-SY5Y cells in the presence of initial and oxidized copolymers of PO and EO. Concentration of rhodamine - 5 µM, copolymers - 1 mg/mL. Values are presented as mean ± SD.

Fig. 4. Representative LSCM images of cross sections of rat spinal cord (Th13 segment) exposed to 2 % polyacrylate gel containing (A) 50 µM rhodamine 123 (control) and (B) 50 µM rhodamine 123 + 5 mg/mL L6-COOH. White line confines the area of intensive fluorescence of rhodamine 123 at gel-contacting site. Cell nuclei are stained with DAPI.

Fig. 5. Effect of oxidized copolymer L6-COOH on intraspinal penetration of rhodamine 123. Area-normalized intensity was calculated using imageJ software. Values are presented as mean ± SEM (n = 6; p = 0.007).

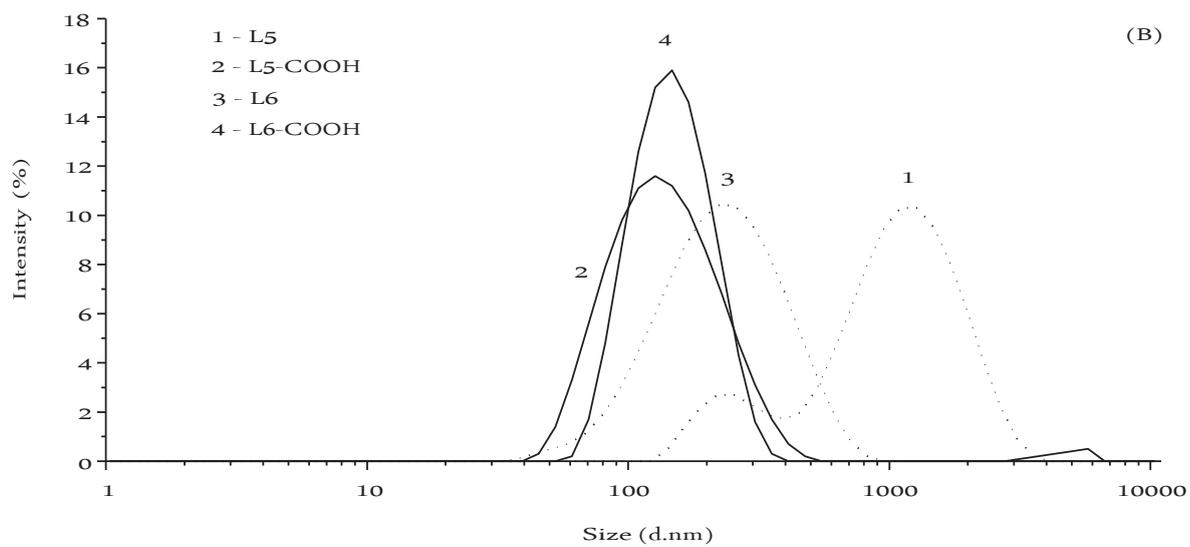
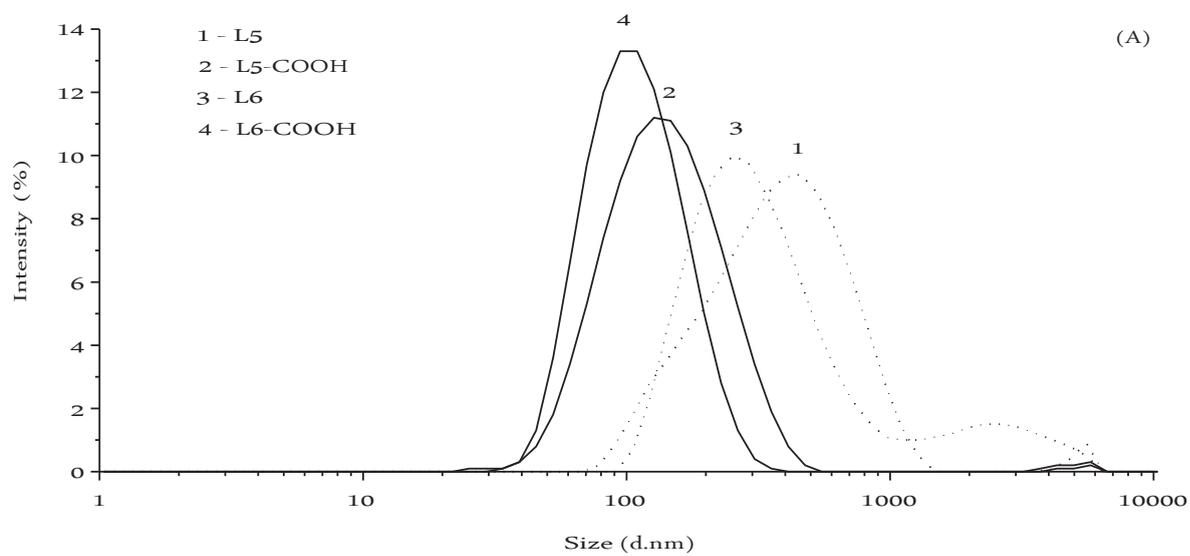


Fig. 1

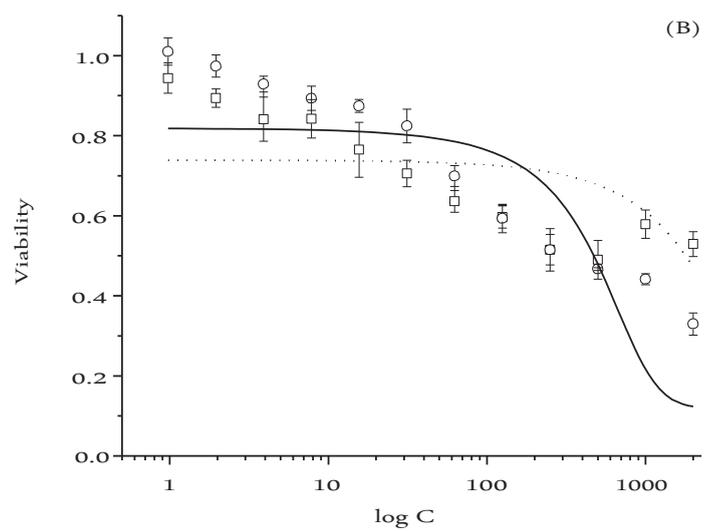
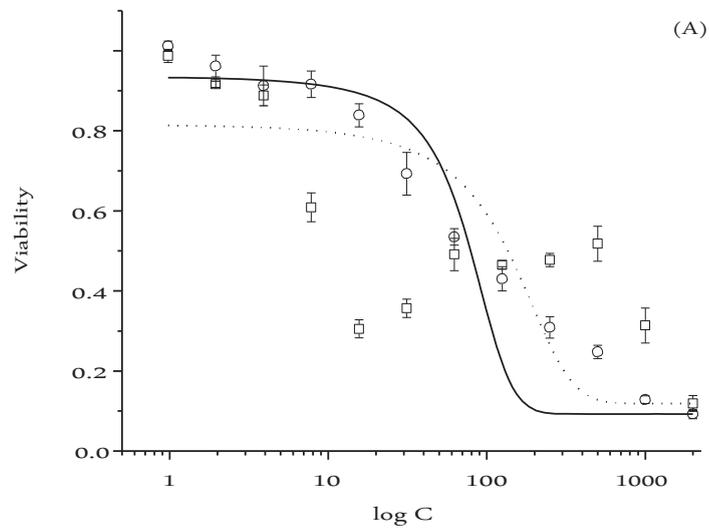


Fig. 2

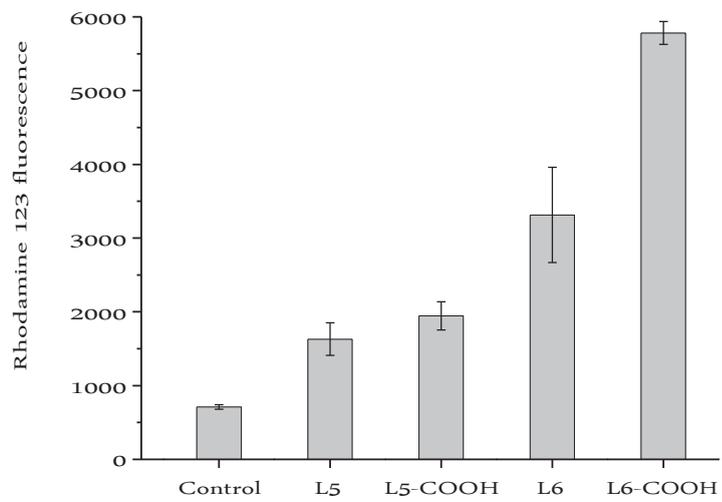


Fig. 3

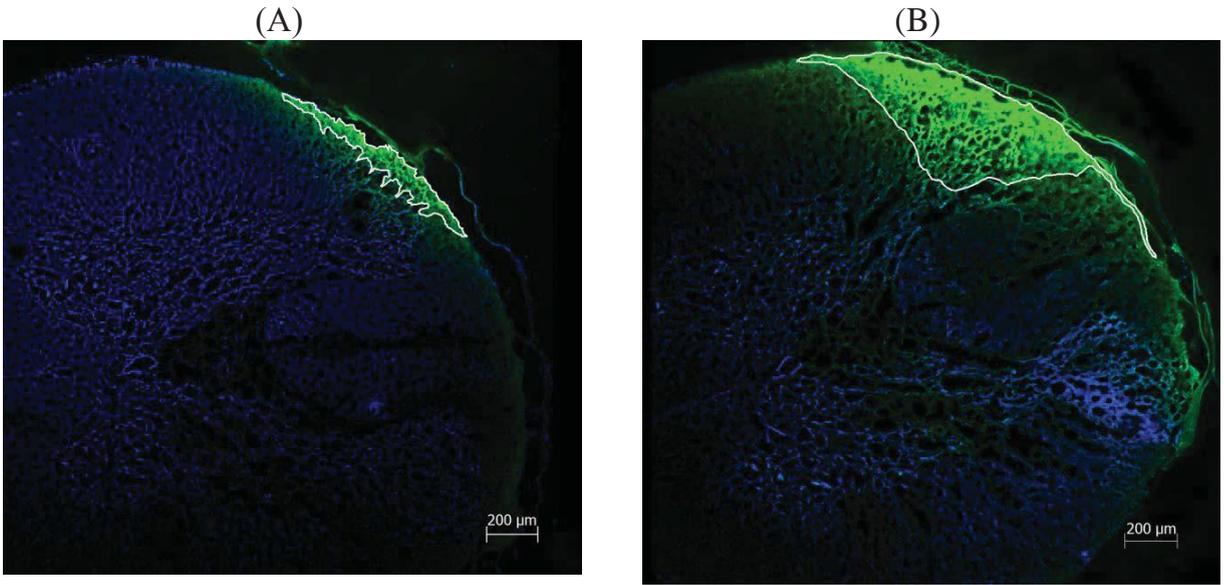


Fig. 4

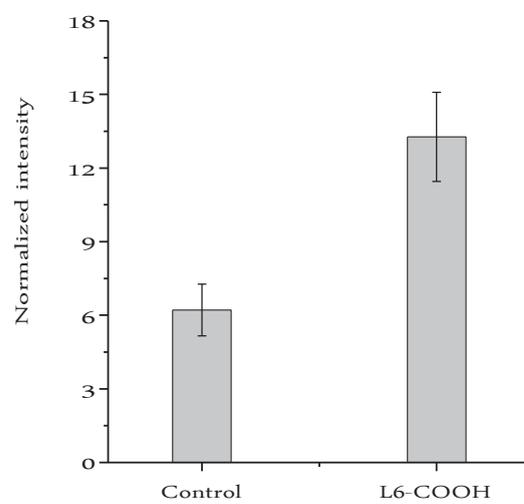


Fig. 5