Journal of Controlled Release 270 (2018) 226–236

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Multilayer polyion complex nanoformulations of superoxide dismutase 1 for acute spinal cord injury

N.V. Nukolova[a,c,1,2], A.D. Aleksashkin[b,1], T.O. Abakumova[a], A.Y. Morozova[a], I.L. Gubskiy[c], E.A. Kirzhanova[b], M.A. Abakumov[c,e], V.P. Chekhonin[a,c], N.L. Klyachko[b,d], A.V. Kabanov[b,d,*]

[a] Department of Fundamental and Applied Neurobiology, V. Serbsky National Medical Research Center of Psychiatry and Narcology, Kropotkinskiy 23, Moscow 119034, Russian Federation

[b] Laboratory of Chemical Design of Bionanomaterials, Faculty of Chemistry, M. V. Lomonosov Moscow State University, Leninskie Gory 1, Moscow 119991, Russian Federation

[c] Department of Medical Nanobiotechnology, N. I. Pirogov Russian National Research Medical University, Ostrovityanova st. 1, Moscow 117997, Russian Federation

[d] Center for Nanotechnology in Drug Delivery, Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, 125 Mason Farm Road, Chapel Hill, NC 27599, United States

[e] Laboratory of Biomedical Nanomaterials, National University of Science and Technology MISiS, Leninskiy prospekt 4, Moscow 119049, Russian Federation

ARTICLE INFO

Keywords:
Antioxidant enzymes
Superoxide dismutase (SOD1)
Spinal cord injury (SCI)
BBB score
Inflammation
Nanoparticles
Double layered polyelectrolyte complex

ABSTRACT

As one of the most devastating forms of trauma, spinal cord injury (SCI) remains a challenging clinical problem. The secondary processes associated with the primary injury, such as overproduction of reactive oxygen species (ROS) and inflammation, lead to concomitant compression of the injured spinal cord and neuronal death. Delivery of copper-zinc superoxide dismutase (SOD1), an efficient ROS scavenger, to the site of injury can mitigate SCI-induced oxidative stress and tissue damage. Towards this goal catalytically active nanoformulations of SOD1 ("nanozymes") are developed as a modality for treatment of SCI. Along with the cross-linked polyion complex of SOD1 with polycation poly(ethylene glycol) (PEG)-polylysine (single-coat (SC) nanozyme), we introduce for the first time the chemically cross-linked multilayer polyion complex in which SOD1 is first incorporated into a polyion complex with polycation, then coated by anionic block copolymer, PEG-polyglutamic acid (double-coat (DC) nanozyme). We developed DC nanozymes with high enzymatic activity and ability to retain and protect SOD1 under physiological conditions. Pharmacokinetic study revealed that DC nanozymes significantly prolonged circulation of active SOD1 in the blood stream compared to free SOD1 or SC nanozymes (half-life was 60 vs 6 min). Single intravenous injection of DC nanozymes (5 kU of SOD1/kg) improved the recovery of locomotor functions in rats with moderate SCI, along with reduction of swelling, concomitant compression of the spinal cord and formation of post-traumatic cysts. Thus, based on the testing in a rodent model the SOD1 DC nanozymes are promising modality for scavenging ROS, decreasing inflammation and edema, and improving recovery after SCI.

1. Introduction

Spinal cord injury (SCI) is one of the most devastating forms of trauma. There are, around the world, between 250,000 and 500,000 new cases of SCI per year [1], most often due to road accidents, falls and violence. Young people make up the majority of the injured population, but regardless of age, SCI tremendously reduces life quality and expectancy. Current treatment of SCI is mostly focused on the minimizing further injury [2,3]. SCI can be divided into three phases: 1) acute phase (or primary injury), that includes fracture, dislocation, damage of blood vessels and nerves, etc.; 2) post-acute secondary phase, that involves multiple pathological mechanisms, such as reactive oxygen species (ROS) formation, neutrophil infiltration, inflammation, etc.; and 3) chronic phase [2,4]. Thus, the secondary processes produce further cellular damage, which can lead to neuronal death and complicate the primary injury.

One of the major inflammatory factors is overproduction of ROS, resulting in the disruption of redox balance, leading to severe damage of DNA, proteins and lipids (lipid peroxidation) [5]. A burst release of ROS at the site of trauma consumes endogenous antioxidants and

* Corresponding author at: Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, 125 Mason Farm Road, Chapel Hill, NC 27599, United States
E-mail address: kabanov@email.unc.edu (A.V. Kabanov).

[1] Equal contribution.

[2] Current address: Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 500 Main Street, Cambridge, MA 02139, United States.

https://doi.org/10.1016/j.jconrel.2017.11.044
Received 12 July 2017; Received in revised form 15 November 2017; Accepted 27 November 2017
Available online 02 December 2017
0168-3659/

affects redox balance. Further, the disturbed balance contributes to uncontrolled peroxidation, inflammation and concomitant swelling [6–8]. The resulting pressure on nerve fibers leads to their degeneration and demyelination (secondary phase of injury). Moreover, the secondary injury process may occur not only at the site of trauma, but also at surrounding tissues, and may last days and weeks [3,4]. Therefore, the burst release of ROS, lipid peroxidation and impaired mitochondrial functions occur soon after the primary injury, whereas the compensatory processes for ROS neutralization start later.

Therapeutic strategies focused on the neutralization of ROS soon after injury could promote the decrease of inflammation and swelling [5,9]. Managing oxidative stress by antioxidants may facilitate recovery and lead to a significant improvement in the state of patients. A number of antioxidants, mainly alpha-tocopherol, ascorbic acid, and ubiquinone, are currently used to control oxidative stress in clinical practice [3,5,9]. Antioxidant enzymes, such as superoxide dismutase 1 (SOD1) and catalase, are highly efficient and can neutralize harmful radicals. In the body SOD1 protects cells from constantly generated radicals and plays the central role in antioxidant defense. Therefore, introducing the antioxidant enzymes after trauma could neutralize the ROS excess and promote more rapid termination of inflammation, thereby lowering post-traumatic neurodegeneration and neuronal cell death [10,11].

However, native enzymes are too vulnerable in the body to benefit from this treatment strategy, because of their rapid elimination and proteolysis; e.g. the plasma half-life of free SOD1 in rats is only 6 min [12]. Several approaches have been developed in order to increase the stability and delivery of antioxidant enzymes in the body. Thus, PEGylation of SOD1 increases the stability of enzymes in circulation to several hours (6 min vs. 36 h), but it limits permeability of enzymes across the brain microvessels [13]. Cationic liposomes loaded with SOD1 have demonstrated the inhibition of infarct volume, although their instability in vivo and potential systemic toxicity impeded their further use [14]. Labhasetwar et al. demonstrated that SOD1 encapsulated in biodegradable poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles reduces the level of the ROS formation and has a protective effect after focal ischemic brain injury [12,15]. Also, suppression of oxidative stress and improved motor recovery of rats after SCI, using lecithinized SOD1 was reported, suggesting the promise of this strategy [16,17].

To address the issue of enzyme stability, previously we have encapsulated antioxidant enzymes into polyion complexes with cationic block copolymers (named “nanozymes”) [18,19]. These nanozymes are stable due electrostatic interactions between the enzyme and polycation block of the copolymer and protect enzymes from the body's proteases. Encapsulated SOD1 was more stable, had prolonged blood circulation and improved the recovery of neurological deficits in animals with various diseases that are associated with uncontrolled release of ROS: angiotensin II induced hypertension, brain damage [18,20].

Despite the seeming simplicity of this approach, the technology efficiency could be greatly improved. For example, to increase the stability of these enzyme-polycation complexes, covalent cross-links were introduced, leading to a significant loss of enzymatic activity [18]. In order to minimally affect the SOD1 activity during cross-linking, a small amount of covalent cross-links was used, resulting in a low protein yield and insufficient retention of SOD1 in nanozymes. Therefore, the synthesis of nanozymes with high enzymatic activity, and high protein yield and SOD1 retention remains challenging.

In the present study, we encapsulated SOD1 into polyion complexes and examined their ability to improve the motor recovery of rats after SCI. SOD1 was protected either by one (polycation) or two (polycation and polyanion) polyion layers, resulting in active and stable single-coat (SC) or double-coat (DC) nanozymes. Using DC nanozymes, we demonstrated the functional improvement in the repair of paralysis in a rodent model of SCI, which was dose dependent. To confirm the recovery of animals, we assessed MRI and immunochemical markers of spinal cord injury.

2. Materials and methods

2.1. Materials

Recombinant human SOD1 was purchased from Life Science Advanced Technologies (Russia). Methoxy-poly(ethylene glycol)$_{113}$-*block*-poly(L-lysine hydrochloride)$_{50}$ (PLL-PEG), poly(L-arginine) hydrochloride (PArg), methoxy-poly(ethylene glycol)$_{113}$-*block*-poly(L-glutamic acid sodium salt)$_{50}$ (PGlu-PEG) were from Alamanda Polymers Inc. (USA). Protamine from salmon, glutaraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), bis-(sulfosuccinimidyl) suberate sodium salt (BS3), quercetin, pyrogallol and Amicon Ultra Centrifugal Filter Units (MWCO 50 kDa) were purchased from Sigma-Aldrich (USA). BCA assay kit and 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) were from Thermo Fisher Scientific (USA). All other chemicals are listed in Supplementary materials.

2.2. Synthesis of cross-linked single-coat (SC) nanozymes

The SC nanozymes were synthesized using a modified procedure of Manickam et al. [18]. Briefly, both SOD1 and PLL-PEG were dissolved in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, pH 7.4, to a concentration 5 mg/ml. The PLL-PEG solution was added to SOD1 solution under gentle mixing to form SOD1-polycation complexes (SOD1:polycation charge ratio was varied from 1:2 to 1:6) and the resulting mixture was kept at + 4 °C for 30 min. The charge ratio was calculated as a ratio of the concentration of NH_2-groups of the block copolymer protonated at pH 7.4 (as indicated by supplier for PLL-PEG) to the concentration of COOH-groups of glutamic acid and aspartic acid residues of the enzyme (estimated using Protein Calculator v3.3 software). Corresponding cross-linkers - DTSSP, EDC, BS3 or GA were dissolved in water and added to the complex to obtain the desired cross-linker feed ratio (a ratio of the concentrations of the added cross-linker and amino groups of PLL-PEG), which was varied from 0.5:1 to 4:1. The reactive mixture was gently stirred overnight at 4 °C. Then, 1 mg/ml $NaBH_4$ solution was added to the nanozymes with GA cross-links (10 μl per 1 mg SOD1) to reduce the Schiff base. To dissociate non-cross-linked polyion complexes the ionic strength was adjusted to 0.3 M NaCl by adding 3 M NaCl and the mixture was further incubated for 10 min. Then the samples were diluted 4 times by adding 10 mM HEPES buffer containing 0.15 M NaCl (HEPES-NaCl buffer, pH 7.4) and purified by serial centrifugation on Amicon filters (50 kDa, HEPES-NaCl, 800 g, 4 °C). Purified samples of cross-linked SC nanozymes were concentrated and stored at 4 °C until further use.

In selected cases, we varied the reaction conditions, in particular, 1) used 10 mM HEPES, HEPES-NaCl buffer and 1 × Phosphate buffered saline (PBS) for formation of polyion complexes; 2) incubation time of complex formation from 30 min to overnight; 3) temperature during a complexation and cross-linking reactions (4 °C or room temperature, r.t.); 4) concentrations of both SOD1 and PLL-PEG from 1 to 5 mg/ml. We also modified the purification procedure of cross-linked complexes by centrifugation using membrane with MWCO 50 kDa (is shown above) or 100 kDa or using size-exclusion chromatography. Sepharose CL-6B column (2 × 50 cm) was equilibrated with HEPES-NaCl buffer (pH 7.4); the samples were monitored by UV at 260 nm (1 ml/min, r.t., HEPES-NaCl) and appropriate fractions were concentrated by centrifugal filtration.

2.3. Synthesis of cross-linked double-coat (DC) nanozymes

The DC nanozymes were synthesized using a two-step procedure. At the first step the polyion complex of SOD1 with a polycation (protamine or polyarginine) was produced. Briefly, 5 mg/ml SOD1 in HEPES-NaCl buffer was supplemented drop-wise with 5 mg/ml corresponding polycation solution in the same buffer to achieve final SOD1:polycation charge ratios 1:2, 1:3 and 1:4 and the resulting solution was incubated