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Superoxide Dismutase-Loaded PLGA Nanoparticles Protect Cultured Human Neurons Under Oxidative Stress

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Abstract The objective of our study was to investigate the neuroprotective efficacy of superoxide dismutase (SOD), loaded in poly(D,L-lactide *co*-glycolide; PLGA) nanoparticles (NPs), in cultured human neurons challenged with hydrogen peroxide (H_2O_2)-induced oxidative stress. We hypothesized that the protected and sustained intracellular delivery of SOD encapsulated in NPs would demonstrate better neuroprotection from oxidative stress than either SOD or pegylated SOD (PEG-SOD) in solution. SOD-NPs (~81±4 nm in diameter, 0.9% *w*/*w* SOD loading) released the encapsulated SOD in an active form with 8.2% cumulative release during the first 24 h, followed by a slower release thereafter. The results demonstrated that PLGA-NPs are compatible with human neurons, and the neuroprotective effect of SOD-NPs is dose-dependent, with efficacy seen at >100 U SOD, and less significant effects at lower doses. Neither SOD (25–200 U) nor PEG-SOD (100 U) in solution demonstrated the neuroprotective effect under similar conditions. The neuroprotective effect of SOD-NPs was seen up to 6 h after H_2O_2-induced oxidative stress, but the effect diminished thereafter. Confocal microscopic studies demonstrated better intracellular neuronal uptake of the encapsulated model protein (fluorescein isothiocyanate-labeled BSA) than the protein in solution. Thus, the mechanism of efficacy of SOD-NPs

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appears to be due to the stability of the encapsulated enzyme and its better neuronal uptake after encapsulation.

Keywords Free radicals · Antioxidant enzymes · Drug delivery · Polymers · Sustained release

Introduction

In recent years, there has been significant interest in developing nanoparticulate systems to overcome the limitations of drug therapy. These nanosystems can improve the therapeutic index of drugs via several mechanisms such as by increasing their stability in the biological environment, enhancing cellular/tissue uptake, providing sustained release, and/or reducing toxicity [1]. Efficient delivery to the brain of high-molecular-weight therapeutics, such as proteins, peptides, and antibodies, has been a challenging goal primarily because of the tight junctions between the brain's endothelial cells that form the blood–brain barrier (BBB) [2]. Furthermore, the efflux action of the membrane-associated P-glycoprotein (P-gp), which is abundantly expressed in the brain's microvascular endothelial cells, prevents the transport of certain drugs into the brain [3]. Therefore, several drug delivery strategies are being explored to facilitate the transport of therapeutic agents across the BBB. Various nanoparticulate systems are being investigated toward this goal, particularly liposomes and drug-loaded polymeric nanoparticles (NPs), that are conjugated to specific antibody or peptide, and have been shown to be effective in delivering therapeutic agents to the brain without affecting the integrity of the BBB [4].

Reactive oxygen species (ROS) are excessively produced in several disease states and their injurious effects contribute to the pathogenesis of many clinical conditions such as atherosclerosis, myocardial infarction, stroke, ischemia/reperfusion injury, chronic and acute inflammatory conditions such as wound healing, central nervous system disorders such as familial amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's dementia [5]. Under normal conditions, a balance is maintained between the formation of ROS by sequential reduction of oxygen during mitochondrial electron transport and the effective removal of ROS by various protective endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). However, under disease conditions, perturbation in mitochondrial metabolism and/or the inflammatory process can cause excessive production of superoxide radicals, which in turn alter the cellular oxidation/reduction balance, resulting in potential oxidative damage [6]. It has been reported that the balance between the production and destruction of ROS depends not only on cellular levels of antioxidant enzymes but also on their relative activities [7]. Several studies have corroborated this finding by reporting decreases in the activities of antioxidant enzymes in different tissues during chronic disease conditions associated with oxidative stress [8].

Exogenous supplementation of antioxidant enzymes has demonstrated therapeutic potential in minimizing the ROS-associated damage [9]. In this study, we have selected SOD, a scavenger of free radicals and a major endogenous cellular defense system against superoxide, for encapsulation in NPs and tested its efficacy in protecting human neurons from hydrogen peroxide (H_2O_2)-induced oxidative stress [10]. Furthermore, SOD mimetics are also known to inhibit several cellular cascades leading to apoptotic cell death [9]. These studies suggest the potential therapeutic role of SOD in treating conditions associated with oxidative stress; however, its use in the form of native enzyme is limited because of its short circulation half-life in vivo (~6 min) and poor permeability across the cell membrane

[6]. Since ROS are known to be present inside cells as well as in the extracellular spaces; therefore, it is essential that anti-oxidant enzymes permeate across the cell membrane for greater efficacy [11]. Different strategies like pegylation, liposomal entrapment, and lecithinization of SOD have been investigated to increase its circulation half-life [12]. Similarly, fusion of transactivator of transcription (TAT)–peptide or tetanus toxin fragment to SOD have been investigated to increase its penetration across the cell membrane [13]. These modifications seem to improve the enzyme activity to some degree over the native form in various models of oxidative stress; however, there are limitations to these modifications. For example, pegylation can increase the stability of the enzyme in vivo (half-life of SOD ~6 min vs. ~30 h of pegylated SOD, PEG-SOD) [14] but can limit its permeation across the cell membrane. Similarly, a fusion peptide such as TAT requires cross-linking to the target protein, which may result in its denaturation and loss of activity [15].

An ideal strategy to effectively neutralize ROS is to successfully deliver antioxidants to inter- and intracellular compartments, as well as to maintain the enzyme activity to neutralize the effect of free radicals that are formed over time. NPs could be an effective drug delivery system for antioxidant enzymes due to their ability to release the encapsulated therapeutic agents in intra- and extracellular compartments at a sustained rate [16]. Therefore, we were interested in testing the efficacy of biodegradable NPs formulated using poly(D,L-lactide *co*-glycolide; PLGA) polymer. We have selected PLGA polymer because of its biocompatibility and biodegradable nature, and it is approved by the U.S. Food and Drug Administration for human use [1].

Our hypothesis was that the protected and sustained delivery of SOD encapsulated in NPs (SOD-NPs) would demonstrate better neuroprotection from oxidative stress than SOD or PEG-SOD in solution. Thus, the goals of the present study were (1) to determine biocompatibility of PLGA-NPs with human neurons, (2) to investigate the dose-response effect of H_2O_2 on neuronal mortality to optimize conditions for oxidative stress, and (3) to evaluate in vitro the neuroprotective efficacy of SOD-NPs in human neurons subjected to oxidative stress.

Methods and Materials

Materials PLGA (MW 23,000; copolymer ratio, 50:50) was purchased from Birmingham Polymers, Inc. (Birmingham, AL, USA). SOD from bovine erythrocytes, PEG-SOD, rat serum albumin (RSA), polyvinyl alcohol (PVA; average MW 30,000–70,000), Tween-80, sodium azide, and fluorescein isothiocyanate-labeled bovine serum albumin (FITC-labeled BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chloroform and all the salts used in the preparation of buffer were purchased from Fisher Scientific (Pittsburgh, PA, USA). Human brain neurons were cultured from materials obtained from the Birth Defects Research Laboratory, University of Washington, Seattle, WA, USA. All studies were performed according to approved guidelines of the National Institutes of Health (NIH) and the University of Nebraska Medical Center.

Formulation of SOD-Loaded NPs Enzyme-loaded NPs were formulated using a typical multiple emulsion-solvent evaporation method [17] but with a modified proprietary composition of the polymer designed to facilitate the release of the encapsulated protein in its active form. In a typical procedure, 90 mg of PLGA was dissolved in 3 ml of chloroform to form a polymer solution. An aqueous solution containing 12 mg of SOD

(~50,000 U) and 18 mg of RSA in 300 μl of purified water was emulsified into the polymer solution by vortexing for 1 min, followed by sonication using a microtip probe sonicator for 2 min on an ice bath at 55 W of energy output (XL 2015 Sonicator® ultrasonic processor, Misonix Inc., Farmingdale, NY, USA). The primary emulsion thus formed was further emulsified into an aqueous solution of 2% PVA by sonication as described above to form a multiple emulsion. The multiple emulsion was stirred overnight on a magnetic stir plate at room temperature to allow the evaporation of chloroform. The NPs thus formed were recovered by ultracentrifugation at 30,000 rpm for 20 min at 4 °C (Beckman Optima™ LE-80K, Beckman Instruments, Inc., Palo Alto, CA, USA); NPs were subsequently washed twice with water to remove PVA and any unencapsulated proteins, and then lyophilized for 2 days (Freeze Dryer, VirTis Company, Inc., Gardiner, NY, USA). The supernatant and washing solutions from NP preparation were collected to determine the amount of SOD that was not encapsulated in NPs. Incorporation of RSA into the formulation served to stabilize the enzyme from interfacial inactivation and also to facilitate the release of SOD from NPs [18]. Control NPs were prepared with RSA alone.

Characterization of NPs The NP size was determined by transmission electron microscopy (TEM). For TEM, a drop of NP suspension was placed onto the TEM grid, and NPs were visualized after negative staining with 2% uranyl acetate (Electron Microscopy Services, Ft. Washington, PA, USA) using a Philips 201 TEM (Philips/FEI Inc., Briarcliff Manor, NY, USA). The mean diameter of NPs was determined from seven different TEM fields for each batch of NPs. The average of three different NP batches was used to calculate the mean diameter of NPs. The hydrodynamic diameter of NPs was measured before and after lyophilization using dynamic light scattering (DLS). To measure the diameter of NPs after lyophilization, a suspension of NPs (0.5 mg/ml) in water was prepared by sonication using a microtip probe sonicator for 1 min as above. The particle size before lyophilization of NPs was measured by diluting the sample of NP formulation before the lyophilization step. The particle size was measured at the intensity of 300 kHz using Nicomp™ 380 ZLS (Zeta Potential/ Particle Sizer, Santa Barbara, CA, USA). The same diluted sample was used to measure zeta potential of NPs using Nicomp™.

Analysis of SOD Activity SOD activity was measured using an SOD assay kit (Dojindo Molecular Technologies Inc., Gaithersburg, MD, USA). This assay is based on utilizing Dojindo's highly water-soluble tetrazolium salt, which produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of reduction is linearly related to the level of xanthine oxidase activity, which is inhibited by SOD. A 20-μl aliquot of each sample (n=3) was added to a 96-well plate, followed by addition of the assay reagents to each microplate well containing sample solutions. The plate was incubated at 37 °C for 20 min, and the absorbance was measured at 450 nm using a microplate reader (BT 2000 Microkinetics, Bio-Tek Instruments, Inc., Winooski, VT, USA). The standard plot (0.1 to 200 U/ml) was prepared by diluting SOD in the dilution buffer provided by the manufacturer.

SOD-Loading in NPs To determine SOD-loading into NPs, the supernatant and washing solutions that were collected during the SOD-NP formulation steps were assayed for enzyme activity. SOD-loading into NPs was assessed by an indirect method, i.e., by determining the total amount of SOD added in the formulation and subtracting that which did not get encapsulated. Our previous studies have demonstrated that the direct extraction of proteins from NPs, by dissolving them in an organic solvent and then repeatedly extracting with water or phosphate-buffered saline (PBS) buffer, does not result in complete

recovery of the encapsulated protein. Using vascular endothelial growth factor-loaded NPs, we have previously demonstrated that quantitation of protein loading, as determined by the indirect method, correlates with the total protein released from NPs in vitro [17].

In Vitro Release of SOD from NPs To study the SOD release profile from NPs, we first determined a suitable buffer that would not degrade SOD under the conditions used for the release study. For this purpose, SOD solutions (50 U/ml) were prepared in PBS (0.15 M, pH 7.4), both with and without the stabilizing agents of RSA (0.1%), Tween-80 (0.05%), and sodium azide (0.05%). These solutions were incubated under the conditions used for the release study, i.e., at 37 °C on a shaker rotating at 110 rpm (Environ®, Lab-line Instruments, Barnstead International Inc, Dubuque, IA, USA). SOD activity was measured over 7 days using the assay as described above. The release study was conducted using double-diffusion chambers as described in our previous studies [17]. Briefly, 6 mg of SOD-NPs were dispersed in 7.5 ml of PBS buffer. For uniform dispersion, the NP suspension was vortexed for 1 min and then sonicated in a water bath sonicator (FS-30, Fisher Scientific) for 10 min. The donor chambers of the diffusion cells were filled with 2.5 ml of NP dispersion (2 mg of SOD-NPs), and the receiver chambers were filled with an equal volume of buffer alone. A Millipore® low-protein binding membrane (Type VV, Millipore Co., Bedford, MA, USA) with 0.1-μm porosity was placed between the two chambers. The membrane is permeable to the protein but not to NPs because the hydrodynamic diameter of NPs is greater than the membrane porosity. The chambers were placed on a shaker rotating at 110 rpm and maintained at 37 °C. The solution from the receiver chambers was removed completely at predetermined time intervals and replaced with fresh buffer. As the buffer from the receiver chambers was removed only at each time point, the protein left in the donor chamber was taken into consideration while calculating the cumulative percent of protein released from NPs.

Cell Culture Primary cultures of human fetal neurons were isolated from the brain tissue of first-/second-trimester abortus materials. The tissue was gathered from the Birth Defects Research Laboratory at the University of Washington, Seattle. This laboratory, which has been funded continuously by the NIH for nearly 40 years, is in compliance with all relevant State and Federal regulations and is approved by the University of Washington's Institutional Review Board. When abortus materials were available, samples of brain tissue (1 to 2 g) were dispensed in 15-ml tissue-culture bottles, each containing 5 ml of culture medium, and were shipped overnight to the laboratory in the Center for Neurovirology and Neurodegenerative Disorders, University of Nebraska Medical Center, Omaha, for preparation of cells. As noted above, the study was performed after approved guidelines of the NIH and the University of Nebraska Medical Center.

Neurons were isolated from the brain tissue using the following protocol. Briefly, the dissociated tissue was incubated with 0.25% trypsin for 30 min, neutralized with 10% fetal bovine serum, and further dissociated by trituration. The resulting single-cell suspension was cultured at 100,000 cells per well on 48-well, poly-D-lysine-coated plates in serum-free, neurobasal culture medium containing 0.5 mM of glutamine, 25 mM of glutamate, 50 mg/ml each of both penicillin and streptomycin, and supplemented with B27 (Life Technologies, Gaithersburg, MD). Half of the culture medium was exchanged every 3–4 days, and neurons were ready for experimental use after 1 week. The purity of the isolated cells was assessed using antibodies against neuron-specific microtubule-associated protein-2 (MAP-2; Boehringer Mannheim Corp., Indianapolis, IN, USA) and glial fibrillary acidic protein (GFAP; Dako Corp., Carpinteria, CA, USA) for detection of neurons and astrocytes,

respectively. This procedure usually results in >85% enriched neurons that are MAP-2 immunopositive.

Cytocompatibility of NPs with Human Neurons Human neurons were incubated for 6 h with control NPs at different concentrations (1 to 8 mg/ml), and in a separate set of experiments, cells were incubated for different time periods (6, 12, and 24 h) with control NPs at 1 mg/ml concentration. A stock dispersion of uniformly distributed NPs (16 mg/ml) was prepared in culture medium. Different doses of NPs (1 to 8 mg/ml) were prepared from the above stock with appropriate dilutions in the culture medium. From each well of the neuronal culture plate, 500 μl of medium was removed out of the 1 ml present, and the dispersion (500 μl) containing the respective dose of NPs was added to the wells and gently mixed. Not more than half of the original culture volume can be replaced without affecting neuronal viability as cells are conditioned to this culture environment. At the end of each incubation time point, 500 μl of the conditioned medium was removed from each well and 50 μl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay reagent (Promega, Madison, WI, USA) was added to each well; medium alone served as a control. The plate was incubated for 3 h at 37 °C in an incubator. The medium from each well was removed carefully without disturbing the formazan crystals present, and then 200 μl of dimethylsulfoxide (DMSO) was added to each well and mixed by gently shaking the plate to dissolve the formazan crystals. The plate was incubated at 37 °C for 5 min, and then 100 μl of the supernatant from each well was transferred to a 96-well plate. The absorbance of the solution was measured at 490 nm using a microplate reader.

Neuronal Uptake of NPs For this experiment, NPs were prepared using the identical procedure as described for the formulation of SOD-NPs, except that SOD was replaced with FITC-labeled BSA. Size and zeta potential properties of FITC-labeled BSA-loaded NPs were identical to those of SOD- NPs. Isolated neurons were cultured on coverslips in a 12-well plate at a density of 0.4×10^6 cells per well. Cells were incubated in the culture medium for different time intervals (1, 6, and 24 h) with either FITC-labeled BSA-loaded NPs (250 μg/ml) or FITC-labeled BSA solution (250 μg/ml), which acted as a control. After incubation, cells were washed three times with PBS (pH 7.4), and then fixed with 4% paraformaldehyde (Polysciences, Inc., Warrington, PA, USA) solution in PBS for 30 min at room temperature. The fixed cells were mounted in Gel Mount aqueous mounting media (Sigma). Confocal images of neurons were obtained with an LSM 510 meta confocal laser-scanning microscope (Zeiss, Jena, Germany) at an excitation wavelength of 488 nm.

Neuroprotective Effect of SOD-NPs from Oxidative Stress As no previous data are available on the dose-response effect of H_2O_2 on human neurons, we first determined the concentration of H_2O_2 required to induce oxidative stress that would not cause excessive neuronal cell death (<70%). For this purpose, we tested the effect of different doses (10, 25, 50, and 100 μM) of H_2O_2 on neurons and measured cell survival after exposure for 6 h. We used the results of this study to determine the optimal concentration of H_2O_2 sufficient to induce oxidative stress for our remaining studies on the neuroprotective efficacy of SOD-NPs.

A stock dispersion of the NP formulation (2 mg/ml, ~800 U of SOD) was prepared in culture medium as described above. To obtain the required SOD dose per well, an appropriate dilution of the stock NP suspension was also prepared in culture medium. From each well of the neuronal culture plate, 500 μl of the medium was removed from the 1 ml present and thereby replaced with a 500 μl dispersion of NPs containing different doses of SOD. In the first set of experiments, either SOD-NPs equivalent to different doses of SOD

(25–200 U/ml) or equivalent doses of SOD in solution prepared in medium were added to the culture plate along with H_2O_2 at the concentration optimized in the above experiment. In the second set of experiments, the neuroprotective efficacy was compared between SOD-NPs and PEG-SOD dissolved in water. In the third set of experiments, the neuroprotective effect of SOD was determined at different time points (6, 12, and 24 h) after induction of oxidative stress. Control NPs (without SOD but containing RSA) and medium acted as the respective controls for SOD-NPs and SOD or PEG-SOD in solution. It should be noted that all the above groups of experiments could not be carried out at the same time because of the varying availability of human fetal brain tissue at different times and the limitation that the cultured neuronal cells have ideal experimental assessments at ~2–4 weeks post-cultivation. The neuroprotective efficacy of SOD was determined using the MTT assay after incubation at 37 °C of culture plates for the above-specified time periods.

Statistical Analysis The two-tailed unpaired Student's *t* test was used to analyze the significance of differences in mean responses between the various treatment groups. Differences were accepted as significant at P values <0.05.

Results

Formulation and Characterization of SOD-NPs The mean size of SOD-NPs measured using TEM was 81±4 nm, and particles were mostly spherical in shape (Fig. 1A). The mean hydrodynamic diameter of SOD-NPs as measured by DLS was greater than the TEM diameter. Before lyophilization, the mean diameter of SOD-NPs was 259 nm [polydispersity index (PI)=0.04], whereas after lyophilization, it was 291 nm (PI=0.12; Fig. 1B). The slight increase in hydrodynamic particle size observed post-lyophilization could be due to partial particle aggregation; however, there was no difference in the zeta potential of SOD-NPs measured before and after lyophilization (−24.5±1.8 vs. −23.12±1.8 mV). Discrepancy between the particle size measured using TEM compared to DLS has been reported in our previous studies and has been attributed to the hydration of the PVA that remains associated with NPs [19].

The standard plot for the SOD activity was linear in the concentration range of 1 to 80 U/ml (R^2=0.9845), and hence, the samples collected to determine the encapsulation efficiency of SOD in NPs and those from the release study were diluted accordingly so that their readings were within the linear range of the standard curve. Based on the amount of SOD that was added and the amount that was not encapsulated, the efficiency of encapsulating SOD in NPs was 75±3.6% (n=3), yielding 75% entrapment of the added protein into NPs. Therefore, the SOD-NP formulation contained approximately 90 μg of SOD per mg of NPs, which was equivalent to 400 U of the enzymatic activity.

The stability study demonstrated that the SOD enzyme activity was retained for more than 7 days in the PBS buffer that contained stabilizing agents (RSA, Tween-80, and sodium azide). However, there was complete loss of enzyme activity in the buffer that lacked stabilizing agents; the activity was initially reduced by 5% during the first 3 days, followed by a rapid loss, with no activity remaining after 5 days (Fig. 1C). Hence, the buffer containing stabilizing agents was used to study the release of enzyme from NPs, as it was additionally confirmed that these agents would not affect the enzyme assay. This in vitro experiment demonstrated the release of encapsulated SOD in its active form, with 8.2±1.6% cumulative release occurring in 24 h and 78±9.3% in about 90 days. The release rate

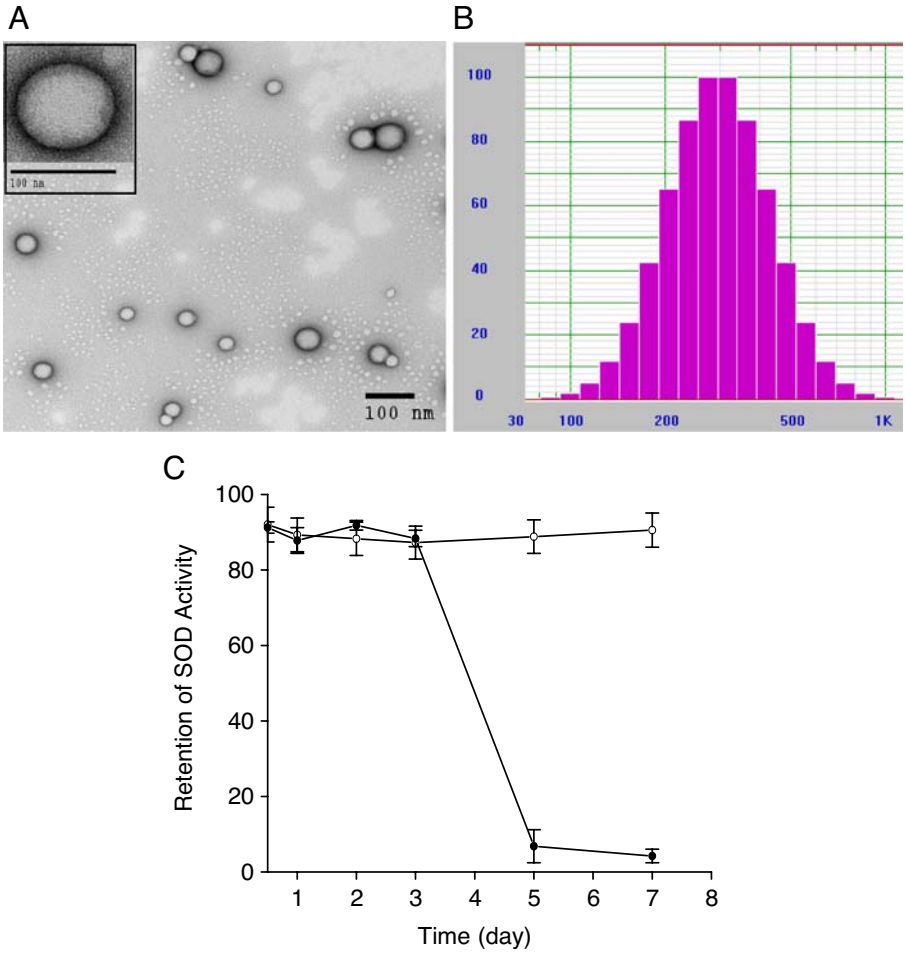

Fig. 1 Physical characterization of SOD-NPs and stability of SOD in solution. **A** Transmission electron micrograph of SOD-NPs. *Bar* represents 100 nm; **B** particle size distribution of SOD-NPs measured using DLS. NPs were lyophilized and resuspended in water before particle size analysis, and **C** stability of SOD solution in PBS without (closed-circles) and with RSA, Tween-80, and sodium azide (open-circles) at 37 °C, mean ± SEM (n=3)

was higher during the initial phase of the study due to the burst release, which is a common phenomenon with PLGA-based particles.

To ensure that there was no loss of H_2O_2 due to its adsorption on NPs in the NP-treated groups, we measured the amount of H_2O_2 in the presence and absence of NPs. First, the optical densities (ODs) of H_2O_2 solutions in water in the concentration range of 0.1 to 100 μM were measured at 240 nm using an ultraviolet spectrophotometer (UV-1601PC, UV-visible spectrophotometer, Shimadzu Scientific Instruments Inc., Columbia, MD, USA) [20]. The standard plot was linear in the concentration range of 0.5 to 5 μM H_2O_2, and hence, we could not use the concentration of H_2O_2 (50 μM) that was used for inducing oxidative stress in cell culture. Therefore, 1 μM H_2O_2 concentration was used to determine the loss of H_2O_2 in the presence of NPs (1 mg/ml). A stock dispersion of control NPs (10 mg/ml) was prepared in water by vortexing and sonication as described previously. A

stock solution of H_2O_2 (10 μM/ml) was prepared in water. In the first experiment, a 100-μl aliquot of NP dispersion in a 1.5-ml cuvet was diluted to 1 ml with water and mixed, and the OD of the dispersion was measured at 240 nm, with water acting as a blank. The NP dispersion did not show any reading at the above concentration. In the second experiment, 100 μl of the NP stock dispersion was mixed with 100 μl of the H_2O_2 stock solution, the volume was adjusted to 1 ml with water, and the OD of the sample was measured, as stated above, both immediately and after 6 h of incubation at 37 °C, with H_2O_2 diluted in water (without NPs) as a blank. There was no difference in absorbance of H_2O_2 when comparing the presence or the absence of NPs and neither when measured immediately after incubation nor 6 h later (0.091±0.001 vs. 0.096±0.006; n=3), suggesting that there was no loss of H_2O_2 in the presence of NPs.

Cytocompatibility of NPs Although the cytocompatibility of PLGA-NPs has been determined in various cell lines, it has not been tested in human neurons, which represent the most biologically relevant in vitro system for the human brain. In the dose–response study, neurons were treated with different doses of control NPs (1 to 8 mg/ml) and incubated for 6 h. In the time–response study, neurons were incubated with 1 mg/ml of control NPs for different time courses (6, 12, and 24 h). The dose– and time–response studies with control NPs did not show any toxic effect in human neurons. Our results showed some variability in cell survival in the time–response study, but the differences between groups were not statistically significant.

Neuronal Uptake of FITC-BSA Loaded NPs The confocal microscopy images demonstrated that the cells incubated with protein in solution displayed no internalization of the protein; rather, there appeared to be an association of protein with the cellular membrane. In contrast, incubation of cells with NPs revealed intracellular delivery of the encapsulated protein. The intracellular fluorescence activity was seen to increase with incubation time in the cells treated with NPs than in the cells treated with protein in solution.

Neuroprotective Effect of SOD-NPs Under H_2O_2-Induced Oxidative Stress The first objective was to determine the concentration of H_2O_2 that would not cause excessive neuronal death (<70%). We found 64% cell mortality at the lowest dose of H_2O_2 (10 μM) tested, which increased marginally to 70% with an increase in the H_2O_2 dose to 50 μM. However, complete neuronal cell death was seen at 100 μM H_2O_2 dose. Therefore, the 50 μM H_2O_2 dose was used to induce oxidative stress and to evaluate the neuroprotective efficacy of SOD. SOD-NPs elicited a dose-dependent response in the survival of neurons after induction of oxidative stress. Maximal neuronal viability occurred between at 100–200 U dose with encapsulated SOD; however, lower doses of SOD-NPs did not demonstrate significant neuroprotection (Fig. 2A). Under the same conditions of oxidative stress, neither SOD (25–200 U) nor PEG-SOD (100 U) in solution were ineffective in demonstrating the neuroprotective effect. When tested at different periods of time (6, 12, and 24 h) after oxidative stress, the neuroprotective effect of SOD-NPs diminished beyond 6 h (Fig. 2B).

Discussion

In this study, we have demonstrated cytocompatibility of PLGA-NPs with human neurons in dose– and time–response studies. Furthermore, we determined the concentration of H_2O_2

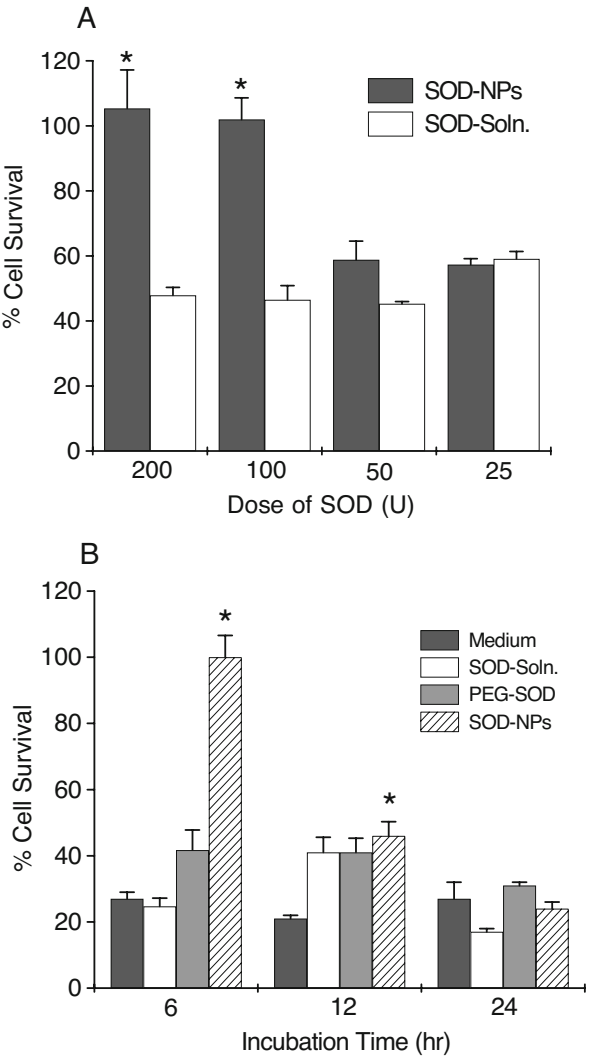

Fig. 2 Neuroprotective efficacy of SOD in human neurons after H_2O_2-induced oxidative stress. **A** Neuronal cells, which were exposed to hydrogen peroxide-induced oxidative stress, were incubated with different doses of SOD either as solution (SOD-Soln) or encapsulated in NPs (SOD-NPs). The resulting cell viability was determined at 6 h using MTT assay; **B** neuroprotective efficacy of SOD at different time points in neurons after H_2O_2-induced oxidative stress and SOD treatment. Dose of SOD=100 U. Data as mean ± SEM (n=3), *P<0.05 against medium control

that could be used to study the effect of oxidative stress in human neurons and demonstrated the efficacy of SOD-NPs in protecting the neurons subjected to H_2O_2-induced, oxidative stress.

As NP-based systems evolve for the delivery of drugs, the issue arises of their biocompatibility with cells and tissue. Though several studies have addressed the compatibility of these NPs in animal cell culture and animal models, it is becoming increasingly important to demonstrate similar results in human cells and tissues to promote their applications in treating human patients [21]. Our studies demonstrated cytocompatibility of NPs at different concentrations of NPs with human neurons. The concentrations of NPs used in our study are higher than what might actually be transported into the brain via systemic administration; however, this high concentration of NPs can be present if administered via direct injection to a specific site in the brain.

Hydrogen peroxide is commonly used to induce oxidative stress in numerous cell lines; however, the response of neuronal cells to the similar doses of H_2O_2 could be different because neurons are especially rich in polyunsaturated fatty acids. For example, in cultured human keratinocytes, H_2O_2 in the dose range of 200 to 700 μM was used to induce oxidative stress; as a result, half of the cells became permeable to trypan blue at 700 μM H_2O_2 dose by 60 min [22]. In our studies with human neurons, we found that a significantly lower dose of H_2O_2 (10 μM) caused 64% cell mortality. Monte et al. [23] observed 42% cell mortality in primary neurons isolated from cerebellar cortical tissue from rat pups at the dose of 90 μM H_2O_2. These observations suggest that the H_2O_2-induced cell mortality depends on the cell line, and it seems that human brain neurons are more susceptible to oxidative stress than neurons isolated from the rat brain.

Our results demonstrated that SOD encapsulated in NPs at ≥100 U provides neuroprotection from H_2O_2-induced oxidative stress. However, this effect was less effective at lower doses of SOD in NPs (Fig. 2A). We did not observe neuroprotection with SOD in solution, which could be due to its rapid degradation in the culture before completely detoxifying free radicals. PEG-SOD in solution was also ineffective in our studies, although several previous studies have reported better antioxidant efficacy of PEG-SOD over free SOD enzyme in different cell lines such as hepatocytes and cardiac myocytes, and also in animal experiments [24, 25]. However, McKinney et al. [26] reported the protective effect of PEG-SOD on endothelial cells yet but not on astrocytes or neuronal plus glial cells in a model of oxidative stress mediated by stretch injury. Therefore, it appears that the protective effect of PEG-SOD could be cell-line-dependent. Although previous studies have reported that PEG-SOD decreases the cerebrovascular permeability, or brain edema, that occurs after ischemia, hypertension, or cold-induced injury, its efficacy in minimizing the ischemia/reperfusion injury in brain is uncertain [27]. We speculate that the protective effect of PEG-SOD seen in the above conditions may be due to the protection of the endothelial lining because of the extended plasma half-life of PEG-SOD over the native form of SOD (~30 min vs. several minutes, respectively) [28] as opposed to neutralization of free radicals formed in neuronal cells. Supporting this notion, there is evidence showing enhanced uptake of PEG-SOD into cultured endothelial cells after 24 h of incubation [29], but there are no reports that have demonstrated increased neuronal uptake of SOD after pegylation [27].

Thus, the improved efficacy of SOD-NPs both over SOD in solution (Fig. 2A) and PEG-SOD seen in our studies could be due to the neuronal uptake of NPs resulting in intracellular release of the encapsulated enzyme, thereby maintaining a protective dose. We have previously shown rapid uptake of PLGA-NPs in different cell lines [1]. Our preliminary studies demonstrated greater neuronal uptake of encapsulated FITC–labeled BSA in NPs than of unencapsulated protein. However, a thorough investigation is needed to demonstrate the increased neuronal SOD activity after encapsulation in NPs, and its ability to neutralize free radicals to elucidate the mechanism of greater efficacy of SOD-NPs over unencapsulated SOD or PEG-SOD. Intracellular delivery of the enzyme is essential to neutralize free radicals because mitochondria are primarily involved in generation of superoxide radicals. The efficacy observed in our studies thus could be because of the ability of NPs to carry the enzyme intracellularly.

Only 6% of the encapsulated SOD (~6 U SOD) was released from NPs (based on the in vitro release data) during the experimental period of 6 h, suggesting that SOD encapsulation significantly enhances the neuroprotective effect from oxidative stress. However, the protective effect of SOD-NPs does not seem to last beyond the initial 6-h time period (Fig. 2B). We speculate that at later time points, there might be depletion of cellular endogenous antioxidants (e.g., catalase), which neutralize H_2O_2 formed as a result of the

catalytic activity of SOD. Under normal physiological conditions, superoxide anion is scavenged by intracellular SOD through conversion into H_2O_2, which is then neutralized by endogenous catalase [6]. However, during oxidative stress, dismutation precedes an overwhelming production of superoxide anion, which leads to accumulation of H_2O_2 due to correspondingly insufficient levels of endogenous catalase [30]. This imbalance may lead to cellular changes in neuronal cells that have undergone sustained H_2O_2 exposure; such alterations are not under the control of the antioxidant effects of SOD. Supporting this argument, it has been reported that systemic administration of SOD increases H_2O_2 not only in neutrophils but also in other cells/tissues that can serve as a secondary messenger in the production of inflammatory cytokines [9]. Thus, the formulation of NPs loaded with SOD and hydrogen-scavenging enzymes like catatase and GPx may offer a better means of neutralizing the H_2O_2 formed as a result of dismutation of free radicals with SOD and perhaps may demonstrate a sustained neuroprotective effect.

Whittemore et al. [31] studied the neuronal changes in cultures obtained from rat embryos with time and dose of H_2O_2 to establish this as a model for oxidative stress. The group reported increased fragmented nuclei in neurons exposed to H_2O_2 for longer than 24 h as compared to neurons exposed for a shorter period. It is suggested that many changes representing oxidative stress such as apoptosis, DNA fragmentation, and influx of Ca^{2+} occur within 3 h of H_2O_2 exposure. Huang et al. [32] used a 45-min exposure to 50 μM H_2O_2 in primary rat cortical neurons, resulting in 40% cell death. Considering the detrimental effect of H_2O_2-induced oxidative stress over a short time period, the protective effect of SOD-NPs observed in our studies of up to 6 h is significant. Most studies have used a shorter H_2O_2 exposure time to determine the protective effect of therapeutic agents in culture and perhaps longer exposure causes changes in the cell membrane and other intracellular organelles, resulting in cell necrosis [31].

Conclusions

Our data demonstrate that the neuroprotective efficacy of SOD-NPs against oxidative stress in cultured human neurons is dose- and time-dependent. We are currently evaluating the efficacy of SOD-encapsulated NPs in oxidative stress-related conditions, such as stroke, where there is excessive production of free radicals after ischemia and reperfusion.

Limitations

Although we have shown the neuroprotective effect of encapsulated SOD, our study also demonstrated that the protective effect does not seem to last beyond 6 h. We are speculating that it could be due to the depletion of cellular endogenous catalase and GPx enzymes, which are essential for neutralizing the damaging effect of H_2O_2 produced during dismutation of superoxide by SOD. The evidence supporting the above assumption would rationalize developing NP formulations with a combination of other antioxidant enzymes to achieve sustained neuroprotective effect. As discussed above, it is also quite possible that the H_2O_2-induced oxidative stress model may not be suitable for testing the efficacy of antioxidant enzyme beyond a few hours. This may be due to other irreversible changes occurring in cells with time after exposure to H_2O_2, which are unrelated to oxidative stress. Nonetheless, a better understanding of molecular mechanism of the protective effect SOD-NPs would further help in developing NP-mediated delivery of anti-oxidant enzymes that

could be effectively used in various conditions of oxidative stress. Unfortunately, limited availability of human neurons precludes our attempt to further investigate all the aspects of the study in this cell line.

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Conflict of Interest M.K.R. and V.L. have financial interest in Telomolecular, Inc.

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