Targeted Delivery of Antioxidant Nanoparticles to Inhibit Restenosis

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Senior Honors Thesis
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4/3/2020

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Abstract

Cardiovascular disease is the leading cause of death globally, often resulting from the development of atherosclerosis. Common surgical interventions, such as balloon angioplasty, repeatedly fail due to the re-narrowing of an artery over time, a process called restenosis. Neointimal hyperplasia, the proliferation and migration of cells that leads to restenosis, is driven by reactive oxygen species (ROS). In natural response to injury, macrophages target inflammation in the vasculature. This response provides a strategy for targeted delivery of therapeutics, but it is unknown whether macrophages can deliver antioxidant enzymes to sites of arterial injury. We hypothesize that macrophages can be loaded with antioxidant enzyme nanoparticles (NPs) to deliver enzymatically active catalase (CAT) or superoxide dismutase (SOD) in order to decrease ROS and hence, inhibit neointimal hyperplasia. Using dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA), catalase NPs and SOD NPs size and dispersity were characterized. Encapsulated enzymes were enzymatically active and stable for 7 days, indicating that nanoparticles do not inhibit enzymatic activity. Macrophage uptake of enzyme-loaded NPs was assessed using fluorescence microscopy. Uptake by murine macrophages (RAW 264.7) peaked at 2 hours with macrophages engulfing more fluorescently-labeled enzyme-loaded NPs than free enzyme. Using macrophages with antioxidant enzyme-loaded nanoparticles is a viable approach to deliver active catalase or SOD, resulting in a targeted delivery system for sites of restenosis. This strategy would present a great advantage to the medical world, as preparation of targeted drug therapy could be executed *ex vivo* with the potential to be implemented back into the patient.

Introduction

Cardiovascular disease is the leading cause of death worldwide,[1] in part due to atherosclerosis. Currently, the techniques used to treat atherosclerosis include angioplasty, stenting, and bypass grafting; however, these interventions can lead to arterial restenosis[2]. Restenosis refers to the re-narrowing of an artery after corrective surgery due to cell proliferation and migration (neointimal hyperplasia) and constrictive remodeling.[2] This event may necessitate further interventions.

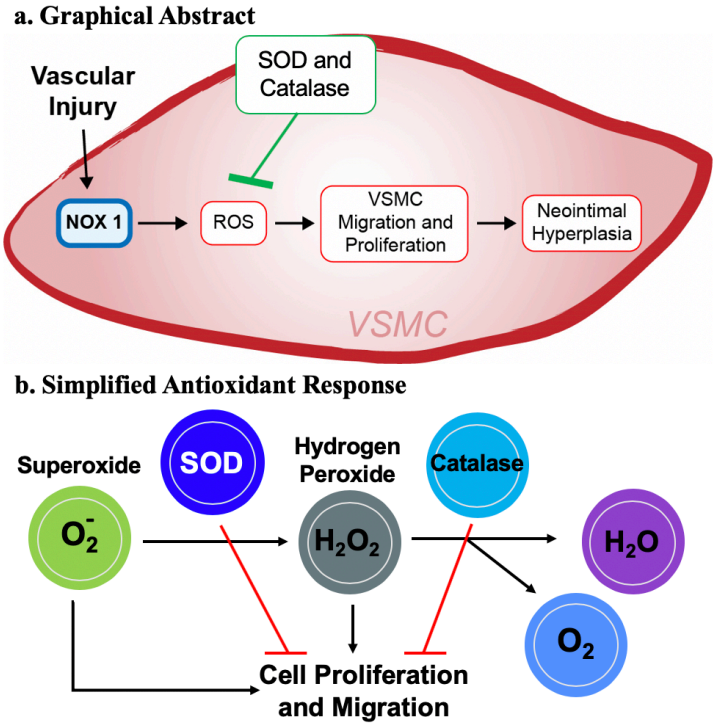


Figure 1: Proposed antioxidant response to inhibit neointimal hyperplasia. **a,** Vascular smooth muscle cell (VSMC) proliferation and migration is mediated by the generation of reactive oxygen species (ROS). SOD1 and catalase can inhibit this production, resulting in the potential to inhibit neointimal hyperplasia **b,** SOD and catalase cause ROS, like superoxide, to form water and oxygen, inhibiting proliferation of VSMCs.

Neointimal hyperplasia is part of an overactive healing response to arterial injury. It is characterized by platelet aggregation and activation, leukocyte migration to the site of injury, extracellular matrix breakdown, and endothelial cell regeneration.[3-5] Overproduction of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, is a characteristic precursor of neointimal hyperplasia.[6] Evidence suggests that reduction of superoxide and hydrogen peroxide levels results in decreased neointimal hyperplasia (Figure 1a).[7,8] Recent studies suggest that the overexpression of antioxidant enzymes, like catalase and superoxide dismutase (SOD), inhibit proliferation of vascular smooth muscle cells *in vitro* (Figure 1b).[8]

It has been shown that enzymes can be delivered to sites of interest via the natural immune response of macrophages. Antioxidant enzymes, such as catalase and SOD, have been delivered using macrophages to sites of inflammation in the brain;[9-13] however, they must be incorporated into nanoparticles in order to avoid degradation and retain activity. We hypothesize that macrophages are recruited to sites of vascular injury and can carry NPs loaded with an antioxidant enzyme (CAT or SOD) to inhibit neointimal hyperplasia.

Nanoparticles (NPs) containing the antioxidant enzymes can be formed by the electrostatic interaction of a positively charged polymer and the negatively charged enzyme at physiological pH. This type of NP provides a polymeric coat around the enzyme, which can preserve the bioactivity of the enzyme until the NP is degraded, enhancing the likelihood that the active enzyme reaches the site of interest.[9-13] Successful inhibition of neointimal hyperplasia via macrophage-mediated delivery of antioxidant NPs would present a great advantage to the cardiovascular field given the translational potential of this therapy.

Results

Nanoparticle Synthesis and Size Characterization

Using electrostatic stabilization of negatively-charged catalase (-252 per tetramer) or SOD enzyme (-38 per dimer) with positively-charged polymer (+50) at pH 7.4, NPs with an antioxidant enzyme core and PolyLysine$_{50}$-PEG (PL$_{50}$-PEG) polymeric corona were produced at a charge ratio of 1. NP formation was confirmed using Transmission Electron Microscopy (TEM) (Figure 2c, f). Catalase-PL$_{50}$-PEG NPs (catalase NPs) and CuZnSOD-PL$_{50}$-PEG NPs (SOD NPs) were analyzed using Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA) to determine size and dispersity. For catalase NPs, DLS analysis reported an average size of 154.7nm +/- 30.8nm and a polydispersity index (PdI) of 0.26 +/- 0.05 (Figure

2b). Additionally, NTA analysis reported an average size of 125.2nm +/- 3.8nm for catalase NPs (Figure 2a). For the SOD NPs, DLS analysis reported an average size of 53.0nm +/- 39.8nm and a PdI of 0.60 +/- 0.2, (Figure 2e). NTA analysis reported an average size of 99.5nm +/- 12.8nm for SOD NPs (Figure 2d).

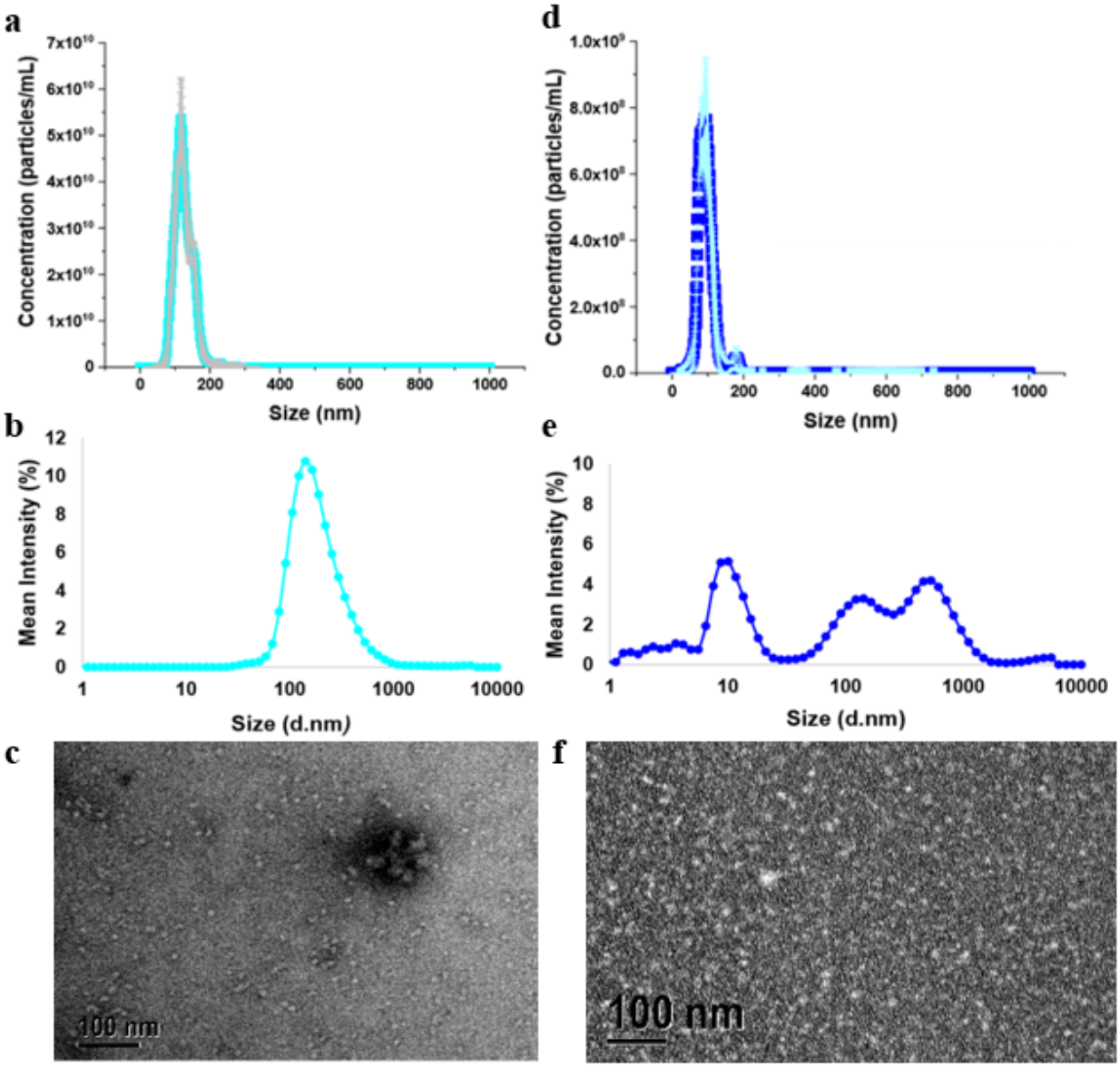


Figure 2. Catalase-PL_{50}-PEG and CuZnSOD-PL_{50}-PEG nanoparticle size characterization. **a,** Catalase NPs (0.001mg/mL) in NTA analysis show an average size of 125.2nm. **b,** Catalase-PL_{50}-PEG NPs (0.01mg/mL) in DLS analysis show an average size of 154.7nm with a PdI of 0.26 at 0 hours. n=9 **c,** Catalase-PL_{50}-PEG NPs can be visualized using TEM. Scale = 100nm. **d,** SOD NPs (0.002mg/mL) in NTA analysis show an average size of 99.5nm. **e,** CuZnSOD-PL_{50}-PEG NPs (0.01mg/mL) in DLS analysis show an average size of 53.0nm with a PdI of 0.60 at 0 hours. **f,** CuZnSOD-PL_{50}-PEG NPs can be visualized using TEM. Scale = 100nm.

Nanoparticle Activity Characterization

Catalase activity was determined by measuring the degradation of hydrogen peroxide into water and oxygen, then the activity of the catalase enzyme in NPs was compared to normal catalase enzyme. Catalase enzyme activity in NPs is comparable to control catalase enzyme function. The enzyme in NPs retains its activity over 168 hours in solution (Figure 3a).

SOD activity was determined by measuring the inhibition of superoxide radical levels using a colorimetric reaction based on a tetrazolium salt, a detailed description of which is in the methods section. SOD enzymatic activity in NPs was compared to free SOD enzyme activity. SOD activity in NPs remains comparable to control SOD enzymatic levels over time. SOD NPs retain enzymatic stability over 168 hours in solution (Figure 3b).

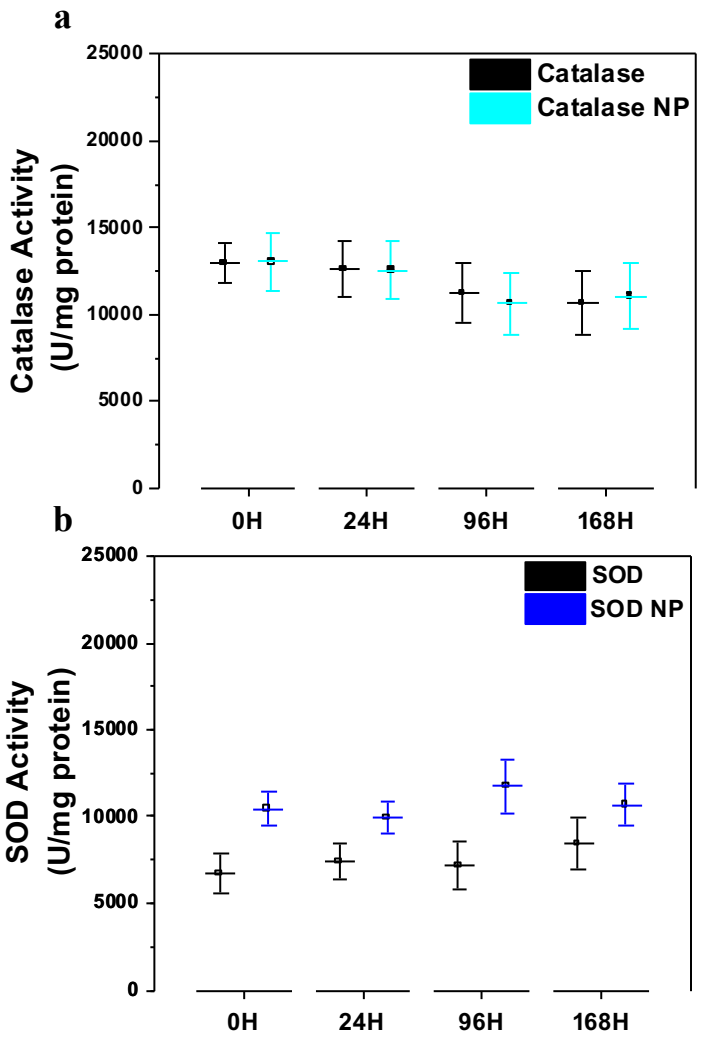


Figure 3. Enzymatic activity over time. **a,** Catalase enzymatic activity remains consistent over 168hrs in both unencapsulated and encapsulated formulations. n=3 **b,** SOD enzymatic activity also remains consistent over 168hrs as free enzyme and NPs. n=3

Nanoparticle Uptake by Macrophages

Ex vivo addition of catalase or SOD NPs to RAW 264.7 murine macrophages resulted in uptake of the NPs (Figure 4). Free enzyme and enzyme NPs were labeled with fluorescein (FITC), macrophage nuclei were labeled with DAPI, and macrophage membranes were labeled

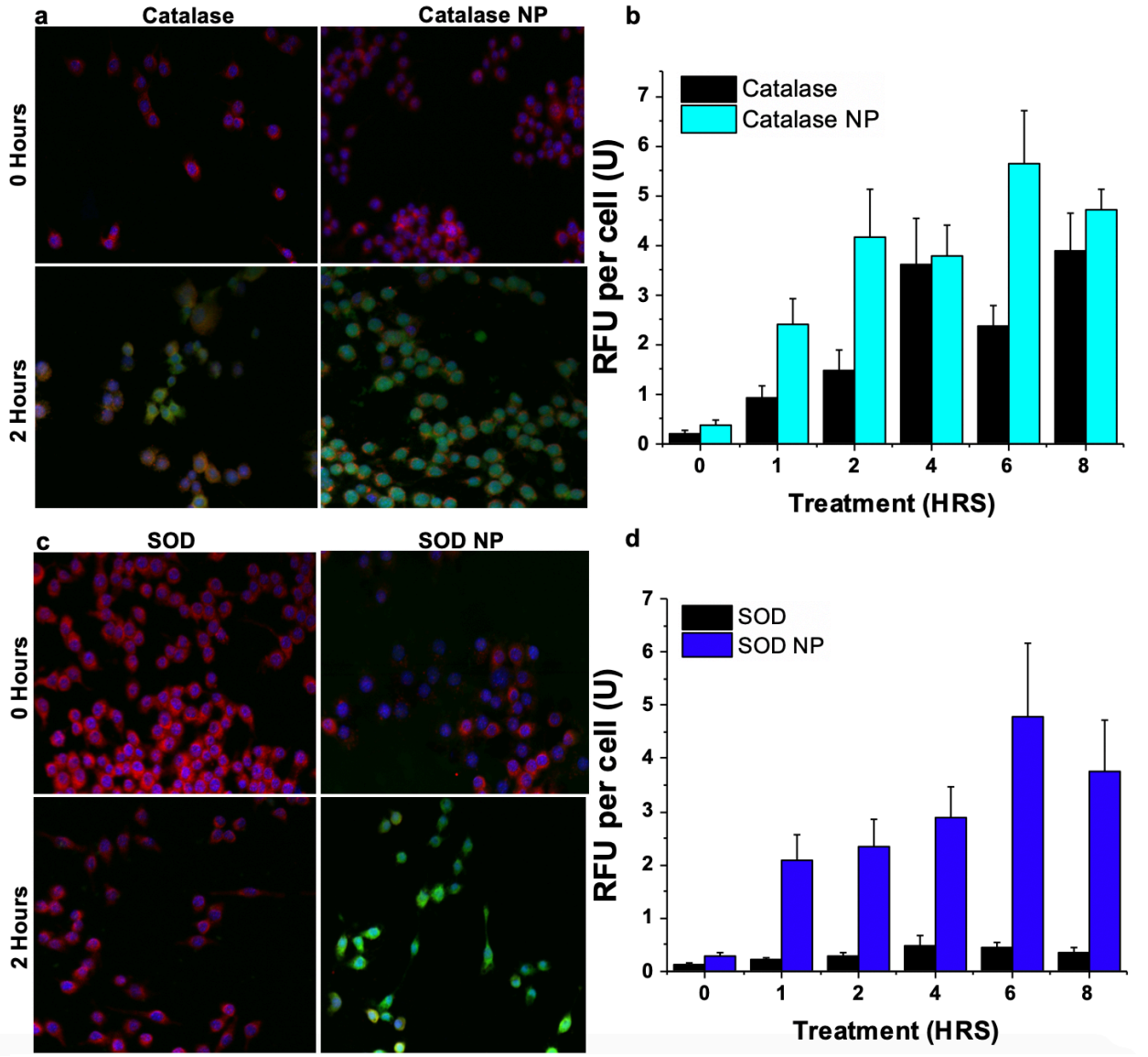


Figure 4. Uptake of catalase or SOD NPs by RAW 264.7 macrophages over time. Enzymes and NPs stained with fluorescence (FITC, green); RAW 264.7 macrophages stained with CDllb (red) and DAPI (blue). **a,** free catalase enzyme and catalase NP uptake by macrophages after 0 and 2 hours of incubation. **b,** relative fluorescence of FITC-labeled cells (NPs) to DAPI-labeled cells (macrophages) for free catalase enzyme and catalase NPs at 0hrs and 2hrs. **c,** free SOD enzyme and SOD NP uptake by macrophages after 0 and 2 hours of incubation. **d,** relative fluorescence of FITC-labeled cells (NPs) to DAPI-labeled cells (macrophages) for free SOD enzyme and SOD NPs at 0hrs and 2hrs. Scale = 20x

with CD11b antibody, a surface receptor marker to identify macrophages. Immediately after adding the particles (0 hours), there was little evidence of NP accumulation in macrophages under any condition. After 2 hours of incubation, SOD NP accumulation in the macrophages was observed, as green fluorescent NPs overlap with red and blue labeled macrophages. The same occurred with catalase NPs. Conversely, free SOD enzyme and catalase enzyme, while present,

were not as robustly localized in the macrophages (Figure 4a, c). Relative fluorescence of FITC-labeled cells to DAPI-labeled cells show that substantial accumulation of both SOD NP and catalase NP into macrophages occurred after 2hrs of incubation, while less accumulation occurred at 0hrs and with free enzyme (Figure 4b, d).

In vivo Nanoparticle Intracarotid Injection

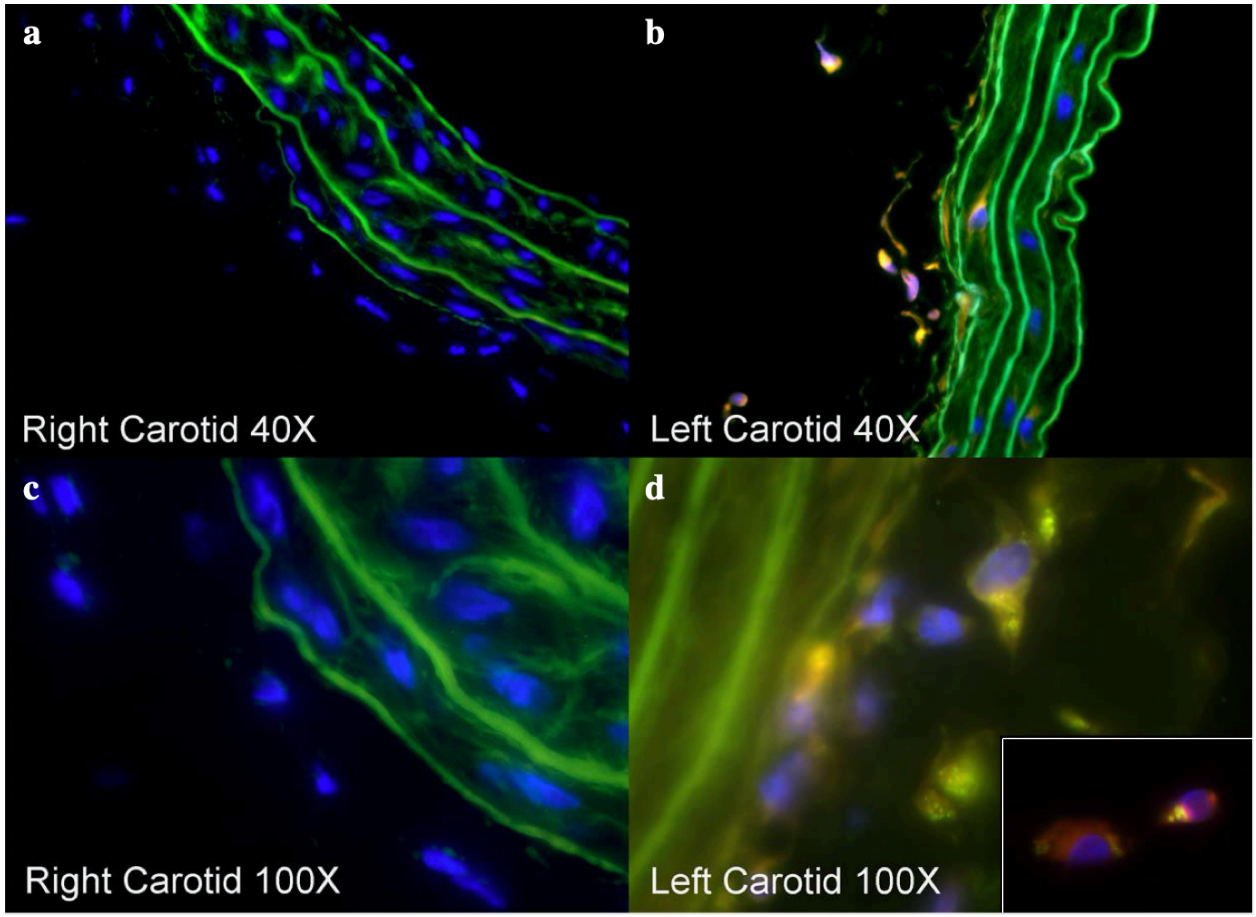


Figure 5. Intracarotid injection of SOD NP-loaded RAW264.7 macrophages to balloon injured carotid artery in a Lean Zucker rat. mKate2-labeled macrophages (red); FITC-labeled nanoparticles (green); cell nuclei stained with DAPI (blue). **a**, uninjured, right carotid artery at 40x scale. **b**, balloon injured, left carotid artery at 40x with accumulation of macrophages (red) and SOD NPs (green). **c**, uninjured, right carotid artery at 100x scale. **d**, balloon injured, left carotid artery at 100x showing colocalization (yellow) of macrophages (red) and SOD NPs (green).

In vivo intracarotid exposure of SOD NP-loaded macrophages resulted in localization of treatment to the injured carotid artery of a Lean Zucker rat (Figure 5). The right carotid artery was uninjured, functioning as a control carotid artery. There appears to be no SOD NP-loaded macrophages remaining in this condition (Figure 5a,c). Conversely, the left carotid artery was

injured through balloon angioplasty surgery, and shows evidence of FITC-labeled SOD NPs (green) colocalized with mKate2-labeled RAW264.7 macrophages (red) remaining around the injured artery (Figure 5b,d). Together, these results demonstrate that SOD NP-loaded macrophages accumulate near the injured carotid artery, but not around the uninjured carotid artery.

Discussion

Throughout these experiments, our results show that catalase and SOD nanoparticles can be formulated by electrostatic interaction between negatively charged enzyme and positively charged PL_{50}-PEG polymer (Figure 2). The catalase or SOD enzyme encapsulated in nanoparticles retain enzymatic activity comparable to that of free enzyme over 7 days (Figure 3). In our experiments, we showed that RAW264.7 murine macrophages preferentially recognize and uptake catalase and SOD NPs over free enzyme (Figure 4). Intracarotid injection of SOD NP-loaded RAW264.7 macrophages to an injured and uninjured rat model revealed that NPs localized to the injured carotid artery (Figure 5). Our results support our hypothesis that macrophages can uptake antioxidant-loaded NPs and deliver them to sites of arterial injury.

At physiological pH, catalase and SOD are negatively charged, allowing these enzymes to electrostatically interact with positively charged PL_{50}-PEG polymer. The charge interactions allow PL_{50}-PEG to coat the negatively charged enzyme and form a protected particle. Dynamic Light Scattering (DLS) reported that the catalase NPs had an average size of 154.7nm and a polydispersity index (PdI) of 0.26 (Figure 2b). The average size of catalase NPs was reported as 125.2nm by Nanoparticle Tracking Analysis (NTA) (Figure 2a). DLS reported that the SOD NPs had an average size of 53.0nm and a PdI of 0.60 (Figure 2e). A PdI of 0.26 indicates relative monodispersity, while a PdI of 0.60 indicates relative polydispersity, but both are consistent with

the current literature.[14] The average size of SOD NPs was reported as 99.5nm by NTA (Figure 2d). These two methods of characterization show consistent sizes for catalase NP formulation, but less in SOD NP formation. Whereas DLS shows three distinct SOD NP populations at sizes of about 10, 100, and 800nm, NTA shows a relatively monodisperse profile of 99.5nm NPs. This could be due to the fact that NTA has a relative lower limit of 50nm, so it would not be accounting for SOD NPs that are small, which DLS is able to detect. Additionally, NTA requires a more dilute sample than DLS; hence, the bigger size aggregates observed for SOD NP by DLS might disaggregate, contributing to the more monodisperse population observed by NTA.

To ensure that encapsulating the enzymes in nanoparticles does not diminish the desired antioxidant effect, the enzymatic activity of loaded NPs and free enzyme were measured over time. When comparing catalase NPs at 0 hours with free catalase at 0 hours, the activities are around 15,000 U/mg protein. At each successive time point, the activities of catalase NPs and free catalase remain similar. Over time, the enzymatic activity of catalase NPs does not seem to decrease (Figure 3a). The same can be said for SOD NPs compared to free SOD enzyme (Figure 3b). This indicates that encapsulation into the NP does not decrease enzymatic activity and that enzymes will remain active in NP form for up to 7 days in solution at 4°C.

To determine whether catalase and SOD NPs can be taken up by RAW 264.7 macrophages, NP accumulation over time was assessed using fluorescent microscopy and quantified using the ratio of cells positive for NPs to total cells. NPs were labeled with fluorescein, while the macrophage nucleus and plasma membrane were labeled with DAPI and CD11b, respectively. When SOD NPs were incubated with RAW 264.7 macrophages for 0 hours, there was no visual evidence of NP accumulation in macrophages (Figure 4c). Additionally, the relative fluorescence of FITC-labeled cells to DAPI-labeled cells was low in

comparison to the 2-hour timepoints (Figure 4d). At 2 hours, visual evidence of NP accumulation was observed and the relative fluorescence substantially increased, showing that NPs accumulate more than free enzyme (Figure 4c, d). The same evidence was observed with catalase NPs accumulation at 2hrs compared to 0hrs, with more NP accumulation occurring than free catalase enzyme (Figure 4a, b). Evidence of free catalase or SOD enzyme accumulation in macrophages was not as substantial, as the relative fluorescence is consistently lower than that of the NPs (Figure 4b, d). This trend suggests that the NP is a more efficient mechanism for macrophage uptake than free enzyme.

To assess the targeting potential of NP-loaded macrophages to sites of arterial injury, we injected SOD NP-loaded RAW264.7 macrophages into a control and injured carotid artery. Fluorescence microscopy revealed that SOD NP-loaded macrophages remained at the injured carotid artery (Figure 5b,d), but did not remain at the uninjured, control carotid artery (Figure 5a,c). This result suggests that the targeting effect of macrophage carriers was achieved, as hypothesized. The yellow appearance of colocalized FITC-labeled SOD NPs (green) and mKate2-labeled macrophages (red) suggests that the macrophage transported the SOD NPs to the target site, as well (Figure 5b,d). Overall, this *in vivo* assay gives preliminary evidence that the NP-loaded macrophage can target the injured artery preferentially to the uninjured artery.

Further experimentation is needed to reduce the limitations of this study. For example, the toxicity of the nanoparticles in both macrophage carriers and vascular smooth muscle cell targets is being assessed. Additionally, the transferability of nanoparticles from macrophages to vascular smooth muscle cells through co-localization needs to be measured. Finally, more *in vivo* injections are needed to improve the significance of the data.

Conclusion

From this data, we can conclude that the synthesis of NPs through electrostatic stabilization of negatively charged antioxidant enzymes with positively charged PL_{50}-PEG polymer was successful. The formulation of catalase and SOD NPs can be replicated with consistent average sizes. Catalase and SOD retain their enzymatic activity when in NP formulation over 7 days. Catalase and SOD NPs can be engulfed by RAW 264.7 macrophages more effectively than free enzyme. Future work will investigate the *in vivo* application of SOD NP-loaded macrophages to determine their effect in rat carotid arteries after balloon angioplasty surgery. Additionally, the toxicity of the NPs will be assessed in macrophages and other affected tissues.

Methods

Cell Culture: RAW 264.7 immortalized murine macrophages were cultured in high glucose (4.5g/L) Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin and streptomycin antibiotics.

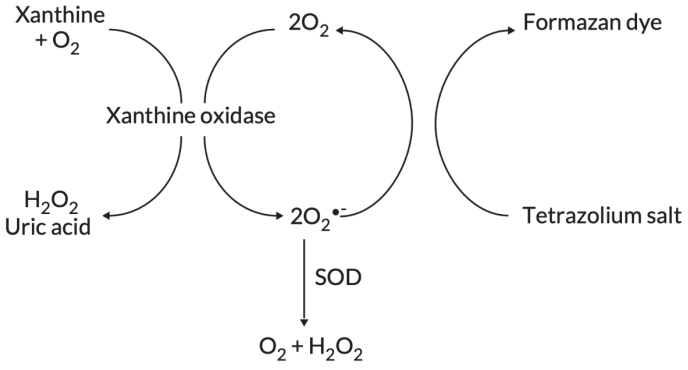
Nanoparticle Synthesis: Catalase (Sigma) and PL_{50}-PEG (Alamanda Polymers) were separately dissolved in 0.2μm-filtered 10mM HEPES buffered saline (pH 7.4; 10mM HEPES + 150mM NaCl) to a concentration of 5mg/mL. Catalase and SOD were then labeled with fluorescein isothiocyanate (FITC, ThermoFisher Scientific) following the protocol outlined by ThermoFisher Scientific. Precalculated amounts of polymer solution were added to the enzyme solution dropwise with gentle vortexing to achieve the desired charge ratio (ratio of the positive charges in polymer to negative charges in enzyme) and enzyme concentration. For example, a 2.5mg/mL catalase enzyme concentration with a charge ratio of 1 required 1.47mL buffer solution, 2mL catalase (5mg/mL), and 530.5μL of polymer (5mg/mL) added dropwise. The solution obtained

was then incubated at 4°C for 30 minutes prior to filtering through a 0.1μm pore size filter for sterilization. CuZnSOD (Sigma) NPs were obtained following the same protocol using 0.2μm-filtered 10mM HEPES buffer.

Dynamic Light Scattering (DLS): Intensity-mean z-averaged particle diameter and polydispersity index (PDI) of the NPs was determined in a Zetasizer Nano S (Malvern Instruments) using a backscatter angle of 173°. Samples were prepared in 0.2μm-filtered 10mM HEPES buffer.

Nanoparticle Tracking Analysis (NTA): A Nanosight NTA (Malvern) was used to analyze the size and size distribution of particles in the catalase and SOD nanoparticle samples. The synthesized nanoparticles were diluted to a protein concentration between 0.001-0.002mg/mL in 0.2μm-filtered 10mM HEPES buffer. The NTA was primed with the same buffer.

Enzymatic Activity: A small volume of catalase only or catalase-PL_{50}-PEG NPs was added to 8mM H_2O_2 in 1mL 50mM phosphate buffer and the catalase-mediated degradation of hydrogen peroxide was measured at 240nm using a spectrophotometer (Molecular Devices). CuZnSOD only and CuZnSOD-PL_{50}-PEG NP enzymatic activity was measured using a tetrazolium based colorimetric assay (Cayman Chemical). In brief, the assay is based on the ability of superoxide radicals to reduce the salt, which goes from yellow to colorless, a schematic of which can be seen on the right. The degree of this change was measured at 460nm in a microplate reader (Biotek) and calculations were performed following the protocol in Cayman's Superoxide Dismutase Assay Kit.



Nanoparticle Uptake by Macrophages: RAW 264.7 macrophages were seeded at a density of $2.5x10^4$ cells per well in a 16-well LabTek chamber slide and allowed to attach overnight. FITC-labeled NPs and enzymes were diluted in media to 0.5 mg/mL enzyme concentration and added to the appropriate chamber. Treatment was removed after two hours. Cells were then fixed with 2% paraformaldehyde for 30 minutes at room temperature. Primary antibody to a pan-macrophage surface marker CD11b (NB110-89474, Novus Biologicals) was diluted in IHC-Tek solution 1:400 and incubated at 4^0C overnight before addition of secondary antibody (A-21245, Invitrogen) at a 1:500 dilution for 2 hours at room temperature. Finally, slides were mounted in DAPI containing mounting media (Invitrogen) and left to set overnight. All steps were preceded by 3X HBSS washes. Images were obtained in a Zeiss LSM 780 confocal microscope at a 20X magnification by another member of the lab. The relative fluorescence of FITC-labeled cells to DAPI-labeled cells was compared using a BioTek Cytation Cell Imaging Reader.

Intracarotid Injection of SOD NP-loaded Macrophages: Our lab technician performed a 5-minute exposure via intracarotid injection of RAW264.7 macrophages loaded with 0.5mg/mL FITC-labeled SOD NPs after catheter removal during balloon angioplasty surgery in a lean Zucker rat following an existing lab protocol.[15] Another member of the lab harvested the left and right carotid artery and stored them in 30% sucrose at 4°C overnight. The arteries were then frozen in Optimal Cutting Temperature (O.C.T.) compound and sectioned using a cryostat. Approximately 2mm of arterial tissue was sectioned in single 5μm cuts, which were mounted on microscope slides beginning at the bifurcation. The sections were then stained with DAPI (20μg/mL) for 10 minutes at room temperature and mounted with mounting media overnight.

Acknowledgements

I would like to thank the Bahnson Research Group, the Kibbe Lab, and Dr. Batrakova for their support and guidance. I would also like to thank Dr. Zachary Nimchuk for his mentoring.

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