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Endothelial targeting of semi-permeable polymer nanocarriers for enzyme therapies

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Abstract

The medical utility of proteins, e.g. therapeutic enzymes, is greatly restricted by their labile nature and inadequate delivery. Most therapeutic enzymes do not accumulate in their targets and are inactivated by proteases. Targeting of enzymes encapsulated into substrate-permeable polymer nano-carriers (PNC) impermeable for proteases might overcome these limitations. To test this hypothesis, we designed endothelial targeted PNC loaded with catalase, an H_2O_2-detoxifying enzyme, and tested if this approach protects against vascular oxidative stress, a pathological process implicated in ischemia–reperfusion and other disease conditions. Encapsulation of catalase (MW 247 kD), peroxidase (MW 42 kD) and xanthine oxidase (XO, MW 300 kD) into ~300 nm diameter PNC composed of co-polymers of polyethylene glycol and poly-lactic/poly-glycolic acid (PEG–PLGA) was in the range ~10% for all enzymes. PNC/catalase and PNC/peroxidase were protected from external proteolysis and exerted enzymatic activity on their PNC diffusible substrates, H_2O_2 and *ortho*-phenylendiamine, whereas activity of encapsulated XO was negligible due to polymer impermeability to the substrate. PNC targeted to platelet-endothelial cell (EC) adhesion molecule-1 delivered active encapsulated catalase to ECs and protected the endothelium against oxidative stress in cell culture and animal studies. Vascular targeting of PNC-loaded detoxifying enzymes may find wide medical applications including management of oxidative stress and other toxicities.

Keywords: Drug delivery; Enzyme; Nanoparticle; Polylactic acid; Antioxidant

Abbreviations: ALI, acute lung injury; BSA, bovine serum albumin; CAM, cell adhesion molecule; DCC, 2, 2-dicyclocarbodiimide; DCF, 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate; DCM, dichloromethane; DLS, dynamic light scattering; EC, endothelial cell; FCS, fetal calf serum; GPC, gel permeation chromatography; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; ID, injected dose; IP, isoelectric point; KRB, Krebs–Ringer buffer; MPEG, methoxy-poly(ethylene glycol); MW, molecular weight; $M \pm$SE, mean plus minus standard error; NHS-biotin, *N*-succinimidyl-biotin; NMR, nuclear magnetic resonance; OPD, *o*-phenylenediamine; PBS, phosphate buffered saline; PECAM, platelet endothelial cell adhesion molecules; PEG, poly(ethylene glycol); PhysProp, physical properties database; PLA, poly(lactic acid); PLGA, poly(lactic-*co*-glycolic acid); PNC, polymer nanocarriers; PSA, polar surface area; REN, human mesothelioma cell line; RPMI, Roswell Park Memorial Institute buffered media; SA, streptavidin; SATA, *N*-succinimidyl-*S*-acetylthioacetate; SMCC, succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate; TBARS, thiobarbituric acid-reactive substances; XO, xanthine oxidase

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1. Introduction

High specificity and potency are the key strengths of protein therapeutics (e.g., enzymes), which have seen an exponential increase in the rate of Food and Drug Administration approval over the past decade [1]. Unfortunately, sub-optimal stability and delivery to therapeutic sites impede the medical use of proteins. Strategies including stealth technologies (i.e., coupling of hydrophilic polyethylene glycol (PEG), that protects cargoes from recognition by host defense systems), designer stabilized enzymes, loading proteins into synthetic depots with controlled release rates [2], synthesis of targeted protein conjugates, fusion and recombinant mutant proteins have all been pursued in an effort to overcome these hurdles [3–6].

The key role and daunting challenges of optimal delivery of therapeutic enzymes is illustrated by the three decades worth of efforts aimed at the important, yet still elusive goal of vascular oxidative stress containment. This pathological condition, induced by reactive oxygen species including H_2O_2 produced by leukocytes and vascular cells themselves, is implicated in acute lung injury (ALI), ischemia–reperfusion, stroke, myocardial infarction, inflammation and other maladies [7]. Antioxidant inducers and high doses of non-enzymatic antioxidants alleviate some forms of chronic oxidative stress, but afford no protection against acute severe insults [8]. Unfortunately, the more potent antioxidant enzymes (e.g., catalase which reduces H_2O_2 to water) have no utility in the treatment of vascular oxidative stress due to poor stability and inadequate delivery to endothelial cells (ECs) lining the vascular lumen [7]. EC represent both a source and a critically important, vulnerable target of oxidants [9–12].

In order to improve endothelial delivery, diverse means have been designed [13,14]. In particular, EC adhesion molecules (CAMs, e.g., platelet-endothelial cell adhesion molecule-1, PECAM-1) represent a good target determinant for delivery of antioxidants and other protein therapeutics for the treatment of vascular maladies [15,16]. PECAM-1 is stably expressed or up-regulated on the surface of endothelium during inflammation and its blockade attenuates leukocyte transmigration [17]. Targeting of catalase conjugated with antibodies against PECAM-1 alleviates acute severe oxidative stress in cell cultures [18,19], perfused organs [20], lung transplantation in rats [13] and oxidative lung injury in mice [21].

Although promising, this approach offers a relatively short (<3 h) duration of protection, due to endothelial proteolysis of conjugates [22], which is sub-optimal for applications requiring more prolonged therapy. In theory, catalase loaded into targeted polymer nano-carriers (PNC) could be more resistant to inactivation. The immobilization of enzymes inside macro- and micro-scale polymeric matrixes protects them from thermal and proteolytic degradation [23,24]. Conceivably, enzymes encapsulated within nanocarriers permeable for their substrates but impermeable to proteases could result in a prolonged therapeutic effect. Previous work of other labs with enzyme reactors designed for industrial use [23,25] that provides high stability of encapsulated proteins [24] lends indirect support for this hypothetical concept.

Catalase is active at the acidic milieu typical of lysosomes and ischemic pathological foci (pH 4–6) and decomposes the highly permeable small oxidant, H_2O_2. Thus, catalase is an attractive candidate for PNC encapsulation. We previously loaded enzymatically active catalase into ~300–400 nm diameter PNC composed of biodegradable di-block copolymers PEG–poly(lactide-*co*-glycolide) (PEG–PLGA) permeable for H_2O_2 and showed that PNC-encapsulated catalase (PNC/catalase) was resistant to proteases and degraded H_2O_2 diffusing through the PNC shell [26]. Based on this initial success, we hypothesized that targeting of PNC/catalase to PECAM would both serve to deliver the antioxidant enzyme to the endothelium and provide sustained protection against vascular oxidative stress. Furthermore, to analyze this platform for vascular detoxification systematically, we loaded a series of other enzymes into PNC and tested their protection against external proteolysis and ability to convert substrates of varying size and hydrophobicity.

2. Methods

2.1. Reagents

Methoxypoly(ethylene glycol) molecular weight (MW) 5000 (mPEG) was obtained from Polysciences (Warrington, PA). Poly(lactic-*co*-glycolic acid)(50:50) in the free acid (38,000 MW) form was from Alkermes, Inc. (Cincinnati, OH). Bovine liver catalase (247,000 MW) was obtained from Calbiochem (EMD Biosciences, San Diego, CA). 10-Acetyl-3,7-dihydroxyphenoxazine, Amplex red®, and Alexa Flour-488-labeled goat anti-mouse antibodies were from Molecular Probes (Eugene, OR). Succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate, *N*-succinimidyl-*S*-acetylthioacetate and *N*-succinimidyl-biotin (SMCC, SATA, NHS-biotin, respectively) were from Pierce Biotechnology (Rockford, IL). $Na^{125}I$ and $Na_2^{51}CrO_4$ were from Perkin Elmer (Boston, MA). Pronase, protease cocktail derived from *Streptomyces griseus*, and all other reagents and solvents were from Sigma–Aldrich (St. Louis, MO).

2.2. Protein iodination

All proteins were radiolabeled with $Na^{125}I$ using the Iodogen (Pierce Biotech., Rockford, IL) method following manufacturer's recommendations, and unbound iodine was removed using gel permeation chromatography (GPC) (Bio-spin 6 Columns, Bio-Rad Labs, Hercules, CA).

2.3. Copolymer synthesis

Two separate methods were used to prepare copolymers for this study. For all polymers, MW and polydispersity index were determined using proton-nuclear magnetic resonance spectroscopy (^1H NMR) and GPC.

mPEG-PLA: D,L-lactide was recrystalized twice in anhydrous ether, and then mixed with mPEG in weight ratios predetermining its MW (20 kDa). The bulk material was raised to 140 °C for 2 h under a purged nitrogen atmosphere, then the temperature was reduced to 120 °C, 1 wt% stannous