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Endothelial targeting of nanocarriers loaded with antioxidant enzymes for protection against vascular oxidative stress and inflammation

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ABSTRACT

Endothelial-targeted delivery of antioxidant enzymes, catalase and superoxide dismutase (SOD), is a promising strategy for protecting organs and tissues from inflammation and oxidative stress. Here we describe Protective Antioxidant Carriers for Endothelial Targeting (PACkET), the first carriers capable of targeted endothelial delivery of both catalase and SOD. PACkET formed through controlled precipitation loaded ~30% enzyme and protected it from proteolytic degradation, whereas attachment of PECAM monoclonal antibodies to surface of the enzyme-loaded carriers, achieved without adversely affecting their stability and functionality, provided targeting. Isotope tracing and microscopy showed that PACkET exhibited specific endothelial binding and internalization *in vitro*. Endothelial targeting of PACkET was validated *in vivo* by specific (vs IgG-control) accumulation in the pulmonary vasculature after intravenous injection achieving 33% of injected dose at 30 min. Catalase loaded PACkET protects endothelial cells from killing by H_2O_2 and alleviated the pulmonary edema and leukocyte infiltration in mouse model of endotoxin-induced lung injury, whereas SOD-loaded PACkET mitigated cytokine-induced endothelial pro-inflammatory activation and endotoxin-induced lung inflammation. These studies indicate that PACkET offers a modular approach for vascular targeting of therapeutic enzymes.

1. Introduction

Biotherapeutics, including enzymes aimed at neutralizing damaging molecular species represent a new, highly promising, and rapidly growing class of potent therapeutic agents. However, their medical utility is impeded by inadequate pharmacokinetics and rapid systemic elimination, suboptimal stability, and other unfavorable factors [1,2]. Furthermore, precise targeting to desired sites at the nanometer scale is hypothesized to potentiate often required for their catalytic functions and enhance the therapeutic effects, while reducing adverse effect. Precise directing of nanodevices to cell-specific targets such as cell adhesion molecules, integrins, and other cell surface antigens via ligand selection (e.g., peptides [3,4], antibodies and their derivatives [4]) enables binding and endocytotic pathway selection [5], potentially directing the biotherapeutic not only to the desired site of action but also shielding it from adverse effects and deactivation [6,7]. Design of carriers that provide site-specific catalytic effects holds promise to improve the utility of this powerful class of biotherapeutics for pharmacotherapy [8–10].

The endothelial cell layer that lines the vascular lumen is an important therapeutic target, in conditions involving oxidative stress and inflammation [11,12]. Excess reactive oxygen species (ROS) cause endothelial damage, dysfunction, and pathological activation that is manifested, among other signs, by the exposure of adhesion molecules (e.g., VCAM-1) which support leukocyte recruitment [13,14]. The vicious cycle of inflammation, oxidative stress, vascular injury, edema, and thrombosis [15–17] propagates disease [17,18], worsens outcomes, and impedes therapeutic management [13,14,19]. Current pharmacotherapy affords no proven protection against dangerous conditions of this nature such as

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acute lung injury (ALI), a prevalent syndrome with unacceptably high mortality and morbidity rates.

The antioxidant enzymes (AOE), superoxide dismutase (SOD) and catalase, are the most potent means to decompose ROS superoxide $O_2^{\bullet -}$ and H_2O_2, respectively. Unfortunately, AOE have limited clinical use, at least in part due to inadequate delivery, characterized by fast elimination, inactivation and lack of targeting. PEGylated, liposomal, and Pluronic-based AOE formulations have prolonged circulation and mitigate ROS in some models of oxidative stress [11]. Yet these formulations have no innate affinity for endothelial cells and do not effectively quench ROS in these cells leaving this therapeutic potential unfulfilled. Targeting approaches using affinity ligands including antibodies to endothelial determinants help to resolve this problem. AOE conjugated with these antibodies (Ab-AOE), exhibit binding and internalization by endothelial cells, necessary for quenching endothelial ROS [20–24]. In animal models of ALI and other forms of acute vascular oxidative stress, Ab-AOEs provide protective effects unmatched by non-targeted AOE and PEG-AOE [21,25,26].

In comparison with the labile Ab-AOE conjugates [7,26], which are degraded in the lysosomes within hours [27,28], AOE encased in carriers permeable to ROS but not proteases offer an additional advantage. Indeed, catalase loaded into such semi-permeable polymeric carriers targeted to endothelial determinant PECAM-1 was resistant to proteolysis [6], accumulated in and protected the endothelium from H_2O_2 for a prolonged time [7].

Superoxide produced in endothelial endosomes [29] mediates pro-inflammatory activation [30] and disruption of endothelial monolayer [31]. Endothelial targeting of SOD-encapsulating carriers may inhibit this pathological mechanism. However, in contrast to neutral H_2O_2, superoxide anion $O_2^{\bullet -}$ poorly diffuses in aliphatic polyester-based polymeric carriers, disqualifying them from SOD delivery. In the first step to attain the goal of extending the range of therapeutically relevant effects achievable via targeted delivery of AOE, we encapsulated catalase and SOD into carriers protecting AOE from proteolysis and permeable for H_2O_2 and superoxide [32]. Here we devised endothelial targeting of these carriers (**Protective Antioxidant Carriers for Endothelial Targeting or PACkET**) as a versatile strategy applicable to both H_2O_2 and superoxide and enabling specific multifaceted protective effects, unattainable by untargeted counterparts.

2. Materials and methods

2.1. Materials

Ferric chloride hexahydrate, ferrous chloride tetrahydrate, sodium oleate (99% pure), Pluronic F-127, xanthine, xanthine oxidase, 2-(N-morpholino) ethane sulfate (MES), Pluronic F127, Celite, and Pronase, whole molecule rat IgG (Rockland) were all purchased from Sigma–Aldrich (St Louis, MO). Radioactive isotopes ^{125}I and ^{51}Cr were purchased from Perkin–Elmer (Wellesley, MA). Catalase and Cu, Zn superoxide dismutase, both from bovine liver, were purchased from Calbiochem (La Jolla, CA). N-Succinimidyl S-acetylthioacetate (SATA), succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), Iodogen and Dylight 488 were purchased from Pierce Biotechnology (Rockford, IL). Mouse anti-PECAM MEC13.3 was purchased from BD Bioscience (San Jose, CA); monoclonal antibody against human anti-PECAM (Ab62) was kindly provided by Dr. Marian Nakada (Centocor, Malvern, PA). Deionized (DI) water (MΩ-cm resistivity) was dispensed by a Millipore water purification system (Millipore, Billerica, MA, USA). Control rat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Enzyme preparation and iodination

Catalase was prepared as previously described [32]. Briefly, catalase was dissolved in deionized water, and then dialyzed in TRIS buffer (pH 7.3) using a slide-a-lyzer dialysis cassette (Thermo Scientific, Rockford IL). Protein concentrations were determined using a standard Bradford assay against a bovine serum albumin (BSA) standard curve [33]. Radiolabeling of catalase, SOD, and antibodies with Na–^{125}I (Perkin–Elmer, Waltham MA) was done as previously described [32] using the iodogen method per manufacturer's description (Pierce Biotech, Rockford, IL). Unbound iodine was removed from the protein solution using gel permeation chromatography (Zeba desalt spin columns, Thermo Scientific). The extent of labeling was verified by a standard trichloroacetic acid (TCA) assay. A 2 μl aliquot of labeled protein was combined with 1 ml of 3% BSA in PBS (pH 7) and 0.2 ml of 100% TCA, vortexed and incubated at RT for 15 min. Precipitated protein was separated from free iodine supernatant by centrifugation (15 min, 4 °C, 2300 rcf) and measured using a Wizard^2 2470 gamma counter (Perkin–Elmer). Catalase was fluorescently labeled with Dylight-488 (Pierce) per the manufacturer's description.

2.3. Biotinylation of Pluronic F-127

The biotinylation of the triblock copolymer Pluronic F-127 was carried out as following (Supplemental Fig. 1). In the first step, Pluronic F-127 was reacted with a large excess of tosyl chloride in chloroform in the presence of triethylamine in an atmosphere of argon at room temperature. The tosylated polymer was coarsely purified from the excess of triethylamine hydrochloride and tosyl chloride via filtration of its toluene solution and several precipitations in hexane, respectively. The resulting tosylated polymer was reacted with the potassium salt of phthalimide in DMF solution at 80–90 °C for 3 h. The phthalimide-modified polymer was coarsely separated from non-polymeric impurities by dissolution in toluene and filtration through a pad of microgranular cellulose (Celite), and purified on a column with silica-gel (eluent $CHCl_3$–MeOH, 1:0 to 1:0.5). Complete modification of the terminal hydroxyl groups was verified by ^{1}H NMR. Integral intensities of signals corresponded to a 1:2 molar ratio of Pluronic F-127 to phthalimide. The phthalimide groups were cleaved using a standard procedure (reflux for 2 h with hydrazine hydrate in ethanol followed by removal of the excess hydrazine in vacuum and acidification with hydrochloric acid). The amino-terminated Pluronic F-127 was dissolved in dichloromethane, purified first by filtration through microgranular cellulose and finally on a column with silica-gel (eluent chloroform–methanol, 30:1 to 1:0.5). The hydrochloride form of the polymer was transformed into the free base by treatment with a large excess of sodium carbonate in water–methanol–isopropanol. ^{1}H NMR (400 MHz, $CDCl_3$) of the amino-terminated Pluronic F-127 exhibited NH_2-bound CH_2 at δ 2.83 and no signals of uncleaved phthalimide residues. The amino-terminated Pluronic F-127 was reacted with biotin N-succinimidyl ester as shown in the final step of Supplemental Fig. 1.

2.4. Antibody-streptavidin conjugate preparation

The antibody conjugates (Ab-SA) were prepared as previously described [7]. Briefly, SMCC was used in 40-fold excess at room temperature for 1 h to introduce stable maleimide groups onto SA. Simultaneously, sulfhydryl groups were introduced onto the antibody through primary amine directed chemistry using SATA. A 6-fold excess of SATA was added to the antibodies at room temperature for 30 min to achieve 1 sulfhydryl group per IgG molecule. Acetylated sulfhydryls were deprotected using hydroxylamine (50 mM final concentration) for 2 h, and antibodies were conjugated with activated streptavidin (SA) at 2:1 molar ratio of IgG:SA. Unreacted components were removed at each step using desalting columns (Thermo Scientific Zeba spin columns; Rockford, IL.)

2.5. Nanoparticle synthesis and characterization

PAC were synthesized using the controlled precipitation approach (Fig. 1) [32]. The initial process involves the preparation of nanocrystalline magnetite (iron oxide) formed through co-precipitation of ferrous and ferric chlorides [62.5 and 170 mg, respectively, dissolved in methanol (2.5 ml) and reacted with 5.0 ml of aqueous sodium hydroxide (0.5 M)]. After maturation of iron oxide in 90 °C for 1 min, the solution was washed twice with DI water by magnetic separation of magnetite particles, and resuspended in 5 ml of aqueous solution containing 225 mg sodium oleate under argon followed by 2 cycles of 5 min incubation at 90 °C using a water bath, followed by 5 min sonication at RT. The black, viscous solution was filtered consecutively through a 5 μm and a 0.45 μm sterile membranes. Subsequent formulations steps were carried out aseptically using 0.2 μm-filtered reagents.

To enable attachment of SA modified antibodies to the particle surface as shown in Fig. 1 C, 0.75 ml 0.1 M calcium chloride was added drop-wise to a mixture of 200 μl catalase (10 mg/ml), 0.75 ml ferrofluid obtained as above, and 200 μl of 10% Pluronic F-127 (doped with biotinylated Pluronic F127 at 2 or 5% of total surfactant, as noted). PAC were washed twice by magnetic decantation and resuspended in 0.75 ml of 5% glucose (w/v).

For radiotracing experiments, PACs were prepared to include either a 10% fraction of ^{125}I-rIgG-SA of total Ab on their surface or 10% of ^{125}I-labeled catalase of total catalase incorporated into the particles.

Zeta potential and hydrodynamic diameter of PAC was measured using 90 Plus Particle Sizer and Zeta Potential Analyzer (Brookhaven Instruments, Holtsville, NY). Zeta potential was determined at different pH conditions following PAC dilution 1:1000 in either Tris or MES buffer (pH 7.5 and 5.5, respectively). Particle size distributions and mean hydrodynamic radii of PAC diluted ×200 were derived from the second order diffusion coefficient obtained from the Stokes–Einstein equation. This measure is independent of particle morphology and refractive index and is derived directly from the scattering intensity data.