Contents lists available at ScienceDirect

Biomaterials

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

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

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ARTICLE INFO

Article history:

Received 3 December 2013

Accepted 8 January 2014

Available online xxx

Keywords:

Antioxidant enzymes

In vivo vascular targeting

Platelet endothelial cellular adhesion

molecules

Inflammation

Nanoparticles

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 H₂O₂ 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.

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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 nano-devices 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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 ($>18\text{ M}\Omega\text{-cm}$ 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-alizer 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 μ l 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 Wizard² 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 ^1H 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. ^1H 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 μ m and a 0.45 μ m sterile membranes. Subsequent formulations steps were carried out aseptically using 0.2 μ m-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 μ l catalase (10 mg/ml), 0.75 ml ferrofluid obtained as above, and 200 μ l 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 -rlgG-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 $\times 200$ 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.

Fig. 1. Endothelial targeted antioxidant nanoparticles formation scheme by controlled precipitation. Legend symbols signify as follows: grey dotted line represents oleate anion, and beneath that it surrounds a small black sphere indicating the oleate coated magnetite. Calcium cations that bind to free and magnetite-bound oleate anions are shown as blue spheres, and antioxidant enzymes (AOE; either catalase or SOD) are large black spheres. The amphiphilic block copolymer Pluronic F127 is indicated by a blue (for hydrophilic polyoxyethylene (POE) moieties) and red (for hydrophobic polyoxypropylene (PPE) line) and then below that the dual biotinylation of the molecule is indicated with green spheres on terminal POE groups. The green symbol labeled mAb-SA indicates monoclonal antibody functionalized with streptavidin. Throughout the text "Ab" represents either human or mouse anti-PECAM; Ab62 or Mec13.3 respectively. (A) combination of aqueous suspension of oleate coated magnetite, AOE and Pluronic F127+/- biotinylation with CaCl_2 drives the controlled precipitation and forms 300–400 nm PACs (B) with surface exposed biotinylated PEO chains. Streptavidin modified Ab/IgG are added, binding to biotinylated PACs (C). Unbound fractions are separated by magnetic decantation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.6. Catalase loading and protection of mass and activity

Loading and protection of catalase mass and activity were characterized as previously described [32]. The incorporation of protein in the particles was measured by radiotracing the labeled catalase distribution between the magnetically separated PAC and the external aqueous phase. The percentage loaded is defined as the quotient of the activity in the final sample to that of the original suspension, adjusted for volume changes.

The catalase activity was measured by a standard hydrogen peroxide degradation assay. PBS-buffered 5 mM hydrogen peroxide solution was added to a quartz cuvette (990–998 μl) and the absorbance was read at 242 nm at RT (Cary 50 UV–Vis, Varian, Palo Alto, CA). Catalase-laden PAC were diluted to a final enzyme concentration of ~ 0.01 – 0.50 $\mu\text{g}/\text{ml}$ corresponding to the linear region of the calibration curve where the slope of the decay curve was proportional to the concentration of the catalase added. Two to ten microliters of PAC were added to a total volume of 1.0 ml. The concentration of the hydrogen peroxide was monitored over time. The activity of the catalase was determined from the slope of the decay curve such that one activity unit corresponds to $23(\Delta\text{Abs}/t)$ [34]. The enzymatic activity of SOD was determined using a cytochrome C reduction assay. Briefly, xanthine and xanthine oxidase are combined to produce superoxide anion with cytochrome C acting as an indicating scavenger that competes with SOD. The working solution (0.6 ml) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 20 μM cytochrome C, and 50 μM xanthine. Reaction was initiated by the addition of 10 μl of 0.2 U/ml xanthine oxidase, and the absorbance was monitored at 550 nm.

A proteolysis assay was used to measure the capacity of the PAC to protect the enzyme cargo. PAC aliquots were incubated for 60 min at 37°C with shaking in a 0.2 wt% buffered Pronase, a robust mixture of proteinases that completely digest proteins into individual amino acids. The retained mass and activity of catalase by PAC after proteolysis was determined following PAC separation by centrifugation (20 min, 4°C at 16.1 rcf) using the radioactivity and enzymatic assays described above.

Retention of Pluronic F127 in the washed PAC was assayed using colorimetric PEG assay based on the PEG-barium iodide complex [35]. Prior to the assay, two solutions were prepared: solution A, consisting of 2.4 g of barium chloride, 8.0 ml of 6 M hydrochloric acid, and 32 ml of DI water, and solution B, consisting of 800 mg of potassium iodide, 500 mg of iodine, and 40 ml of DI water. A calibration solution of 20 $\mu\text{g}/\text{ml}$ Pluronic F127 in DI water was serially diluted to a detection range of 0.1–10 μg . The initial solutions, PAC aliquots and wash supernatants were diluted to within the

detection range (PAC by $\times 100$, unwashed samples and supernatants by $\times 500$). Standards and samples were added to a 96 well plate and diluted to a total volume of 170 μl with DI water. 40 μl of solution A was added to each well and mixed thoroughly. A 40 μl of a $5\times$ dilution of Solution B was added to each well and well mixed. After developing for 10 min at RT in darkness, the absorbance was read at 550 nm on a microplate reader (model 2550-UV, Bio-Rad Laboratories, Hercules, CA).

2.7. Antibody to particle binding and retention

The capacity of the biotin functionalized PAC to bind antibodies was measured by radiotracing ^{125}I -rlgG-SA. Antibody-SA radiolabeling was performed using iodination beads per the manufacturer's protocol (Pierce Iodination Beads, Thermo Scientific.) Biotinylated PAC were incubated with varying concentrations of labeled antibodies for 30 min and then were magnetically separated from unbound particles. The number of bound Ab per particle was calculated based on the quotient of the radioactivity in a known protein to radioactivity concentration of the ^{125}I -rlgG-SA to a known particle concentration of PAC (5×10^{11} particles/ml [32]). Retention of the particle-bound antibodies was tested by incubating Ab-PAC aliquots in either pure mouse serum or 5% glucose at 37°C . The aliquots were sampled at indicated times, centrifuged (20 min, 4°C at 16.1 rcf), and the radioactivity in the supernatant and the pellet were measured to determine the release of Ab.

2.8. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were purchased at first passage from Lonza Walkersville (Walkersville, MD), and were grown in Falcon tissue culture flasks (BD Biosciences, San Jose, CA) coated with 1% gelatin (Sigma–Aldrich) in EGM-BulletKit media (Lonza Walkersville) containing 10% v/v fetal bovine serum (FBS). Passages between 4 and 6 were used throughout the studies. Confluent HUVECs (10^5 cells/ cm^2) were used for the binding and endocytosis studies.

2.9. In vitro Ab/IgG PAC binding to endothelial cells

Human anti-PECAM (Ab62)-SA or rlgG-SA conjugated PAC were formulated with either 2% or 5% biotinylated Pluronic F127 relative to total surfactant that included 10% ^{125}I -rlgG-SA (of total Ab) for quantification of particle binding. Purified aliquots of PAC in increasing concentrations (1.0 – 6.0×10^3 PAC/cell) were added to complete EGM media and incubated with live confluent HUVECs grown to confluence in 24

well plates (BD Tissue Plates, Fisher Scientific.) After incubation with targeted or non-specific rIgG control PAC, confluent HUVECs were rinsed and lysed (1% Triton X100 in 1 N NaOH). Radioactivity in cell lysate was measured and compared to the amount of total activity (Wizard 1470 gamma counter, Wallac, Oy, Turku, Finland). Particle number bound per cell calculations derived from a PAC concentration of 5×10^{11} #/ml [32] and an EC density of 10^5 cells/well. To visualize binding and investigate cargo vs particle delivery, PECAM-targeted PAC prepared with enzyme fluorescently labeled with Dylight 488 were incubated with HUVECs grown on round glass coverslips 60 min at 37 °C with 5% CO₂. After incubation with PAC, cells were washed with PBS 3×, mounted with Prolong Gold anti-fade reagent with DAPI mounting media (Invitrogen) and imaged on a fluorescent microscope (Nikon Eclipse TE2000-U, Nikon, Tokyo, Japan.) in green and blue channels.

Further fluorescence studies were carried out to evaluate particle uptake. Endocytosis studies were performed with PAC loaded with fluorescently labeled catalase as above. In order to differentiate between surface bound and endocytosed PAC, cells were stained without permeabilization with anti-mIgG-Alexafluor 594. Ab62-PAC and rIgG-modified control PAC were incubated with HUVECs as described. After incubation with PAC, cells were washed, mounted with Prolong Gold anti-fade reagent with DAPI mounting media (Invitrogen) and imaged on a fluorescent microscope (Nikon Eclipse TE2000-U, Nikon, Tokyo, Japan.) in red, green and blue channels. Microscope controlling and image processing were done using Image-Pro Plus 4.5.1.27 software (Media Cybernetics, Bethesda, MD.)

2.10. *In vivo* Ab/IgG PAC distribution

All animal experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. *In vivo* distribution of PAC was performed as described earlier [7]. Mouse anti-PECAM (Mec13.3-SA) PAC were injected intravenously into normal C-57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME). Anesthetized mice were injected intravenously with 10 mg/kg ¹²⁵I-labeled Ab-PAC and IgG PAC. For these experiments PAC coated with approximately 500 Ab/particle were diluted into 0.22 μm-filtered 5% aqueous glucose solution. Blood and organs (heart, kidneys, liver, spleen and lungs) were collected at 30 min post-injection. The radioactivity and weight of the samples were determined to calculate PAC targeting parameters, including percent of injected dose per gram (%ID/g), organ-to-blood ratio (localization ratio), and immunospecificity index.

2.11. Endothelial protection by Ab/IgG-PAC catalase from H₂O₂ oxidant stress *in vitro*

Cell death was determined by the specific release of ⁵¹Cr [34]. Confluent HUVECs in 24 well plates were labeled by the addition of ⁵¹Cr isotope (~activity 200 kcpm/well) in complete media a day prior to H₂O₂ challenge experiments. Cells were rinsed of free isotope then incubated with Ab62-PAC or control IgG PAC with either of two concentrations of catalase, or plain media for 60 min at 37 °C with 5% CO₂, then rinsed three times with serum-free media. H₂O₂ (10 mM in serum free media) was added to all but one subset of cells and maintained for 5 h at 37 °C. Cell media and lysate was collected as previously described in binding studies. The total radioactivity in the supernatant and the cell lysates was measured by gamma counter; the release of ⁵¹Cr was used as a marker of cell death. Protection was defined as the difference between the release of ⁵¹Cr in cells treated with H₂O₂ only and those treated with catalase loaded PAC, corrected by the release of isotope in untreated control cells.

2.12. LPS mitigated pulmonary edematous injury in bronchoalveolar lavage

Lipopolysaccharide (LPS) was administered intratracheally at 1 mg/kg in 50 μl of sterile PBS followed by 100 μl of air to induce acute pulmonary injury 15 min post intrajugular injections of Ab/IgG PAC with loaded with AOE. After 24 h, bronchoalveolar lavage (BAL) was performed by exposing and cannulating the trachea with a 20 gauge angiocatheter (BD Biosciences, Sandy, UT) and then lavaging three times with 0.5 ml of PBS containing a protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) at 10 mg/ml. The lavage fluid was centrifuged at 2000 rpm for 4 min, and the supernatant was collected and frozen at -80 °C. Protein concentration was measured using a standard BCA assay (Pierce Chemicals; Rockford, IL). WBC counts in the BAL fluid were determined from precipitates remaining after removal of the cell-free supernatant by counting known volume aliquots under a light microscope (Olympus CKXY1, Center Valley PA) on a hemocytometer [36].

2.13. *In vitro* and *in vivo* expression of inflammatory marker by western blot analysis

For Western blot cell culture analysis of VCAM in HUVECs, 24 well culture dishes (~10⁵ cells/well) were washed twice with PBS and lysed in 100 μl of sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis. For lung homogenate VCAM detection, lungs of animals exposed to LPS as described treated with Ab/IgG-PAC SOD (1 h) were homogenized in a protease inhibitor in PBS for 6 min with a 5 mm steel ball (TissueLyser II; Qiagen, Valencia, CA), lysed with 10% Triton X100/10% SDS for 1 h rotating at 4 °C, sonicated for 20 s, and then centrifuged for 10 min at 16 krcf at 4 °C. The resulting lung homogenate was removed as supernatant. Lysed cells or lung homogenate were added to a 4–15% gradient gel. Gels were transferred

to a PVDF membrane (Millipore, Billerica, MA) that was subsequently blocked with 3% nonfat dry milk in TBS-T (100 mM Tris, pH 7.5; 150 mM NaCl; and 0.1% Tween 20) for 1 h, followed by incubations with primary and secondary antibodies in blocking solution. The blot was detected using ECL Plus reagents (GE Healthcare, New York, NY). Quantification of blots was done using standard densitometry methods (Biorad Fluor-SM, Biorad Laboratories, Hercules, CA).

2.14. *In vivo* expression of cytokines in mouse lungs exposed to LPS treated with Ab/IgG-PAC-SOD

Mouse lung homogenate of LPS exposed animals treated with Ab/IgG SOD PACs (as described above) were measured for MIP2 and TNF expression using ELISA (Quantikine ELISA Mouse CXCL2/MIP-2, Mouse TNF-? Immunoassay, R&D Systems, Minneapolis MN) using the manufacturer's instructions.

2.15. Statistical analysis

Experimental data were analyzed using a two variable heteroscedastic student *t*-test. Differences were determined significant at *p* < 0.05.

3. Results

3.1. Synthesis and characterization of targeted Protective Antioxidant Carriers (PACKET)

We synthesized the PACKETs using a protocol described for prototype PAC lacking immunotargeting [32]. The ion pairing between magnetite-surface bound and free oleate (Fig. 1A) with Ca²⁺ slowly introduced into the system in the presence of Pluronic F-127 leads to formation of PAC (Fig. 1B). Inclusion of biotinylated Pluronic up to 5% of total Pluronic content did not change PAC properties (Table 1). AOE loading efficiency achieved ~30%; the encapsulated enzyme retained ~30% of catalytic activity and ~25% of the enzyme load was protected from proteolytic degradation. Purified PAC retained ~15% of Pluronic F-127 used during the particle formation step, indicating that ~6 × 10³ and ~1.5 × 10⁴ of biotin residues are included per PAC at 2% and 5% ratios of biotinylated to unmodified Pluronic F-127, respectively (Supplemental Table 1).

For modular coupling to PAC surface via biotin, antibodies (Ab) and control IgG were first conjugated with streptavidin (SA) using bi-functional SATA-MCCC cross-linker pair as described [7]. Resultant covalent Ab/SA and IgG/SA conjugates possess monovalent residual biotin-binding capacity, thus preventing aggregation of biotinylated carriers [7]. After magnetic separation of biotin-PAC from the excess free components, incubation with Ab-SA yields PACKET indicated thereafter as Ab-PAC and control IgG PAC (Fig. 1C). Up to ~250 molecules of IgG/SA conjugate could be coupled per PAC (Fig. 2A), and retained stably bound to PAC in buffer and serum (Fig. 2A). Zeta potential measurements indicate that surface charge of PAC decreased slightly after conjugation of either Ab or control IgG (Supplemental Table 2).

3.2. Endothelial targeting of PACKET

PAC were conjugated to monoclonal antibodies (Ab) to human and mouse PECAM-1. This ubiquitous and stably expressed endothelial determinant is used by several labs for endothelial targeting of enzymes, liposomes and other carriers [37]. PECAM-targeted PAC bind to endothelial cells, whereas control IgG-coated PAC showed

Table 1

Zeta potential (PACKet with catalase)	
Preparation	Zeta potential (mV)
PACKet no Ab	-24.8 ± 1.2
PACKet + IgG	-24.8 ± 1.1
PACKet + Ab62	-31.4 ± 2.9

Fig. 2. Inclusion of biotinylated Pluronic F127 enables mAb-SA binding to pre-formed PACs. (A) Binding of mAb-SA +/- inclusion of biotinylated Pluronic F127. Ab-SA to PAC binding measured through magnetic decantation separated unbound fraction mAbs from PAC-bound mAbs; radioactivity of fractions measured by gamma counter. (B) Stability of Ab-PAC in serum versus 5% glucose. Data shown as percentage of total released over 60 min at 37 °C over time measured as in (A).

minimal binding (Fig. 3A). Fluorescent microscopy of PACKET labeled with the green fluorescent dye confirmed high specificity of endothelial targeting of PACKET (compare Fig. 3B and C), resulting in punctuated pattern of cellular fluorescence, typical of endosomal and lysosomal localization. In support of the notion of effective internalization of PACKET, staining PACKET on the cell surface by secondary antibody showed that ~50% and 80% of cell-bound PACKET become not accessible from the surface at 30 and 60 min incubation, respectively (Fig. 3C & D).

Next, we characterized endothelial targeting in animals. Since the pulmonary vasculature contains ~25–30% of the total endothelial surface in the body and receives >50% of total cardiac blood output, carriers targeted to PECAM and other endothelial determinants are expected to accumulate preferentially in the lungs after intravenous injection [38]. We found that 30 min after intravenous injection of Ab-targeted PACKET in mice $220 \pm 18.9\%$ of injected dose per gram of tissue (corresponding to $33 \pm 2.8\%$ of the total injected dose) accumulated in the lungs (Fig. 4A). In sharp contrast, less than 10% ID/g of control IgG PAC accumulated in the lungs, confirming that the pulmonary uptake is due to affinity targeting, not non-specific uptake such as mechanical retention in the microvasculature (Ab- and IgG-coated PAC formulations have the same hydrodynamic radius).

The accumulation of PACKET in the liver and spleen was reduced as compared to control carriers (Fig. 4). The opposite distribution of targeted vs non-targeted PACKET in the lungs vs reticulo-endothelial system (RES) organs becomes even more obvious after taking into account differences in blood level (Fig. 4B). The ratio of uptake in lung vs liver achieves 4.3 vs 0.096 for targeted vs untargeted PACKET, respectively which represents a 45× fold increase of lung to liver specificity (Fig. 4C). Calculation of the immunospecificity index (ISI, a ratio of %ID/g of targeted vs non-targeted carrier in an organ normalized per corresponding blood levels) yielded the pulmonary ISI close to 30, one of the highest reported in the literature.

3.3. Antioxidant protection by PECAM-directed catalase PACKET

The protective effect of catalase PACKET was first tested in endothelial cells exposed to a toxic level of H_2O_2 as a simplified model experiment. Targeted catalase PACKET provided about 75% protection from cell death caused by the ROS, whereas untargeted counterpart provided no significant effect (Fig. 5A). Having affirmed the functional activity of catalase PACKET, we examined its effect *in vivo*, injecting it IV in mice exposed to acute pulmonary inflammatory injury caused by bacterial endotoxin (LPS) instilled in

Fig. 3. Binding of Ab-PAC to cultured endothelial cells. (A) ^{125}I labeled PACs incubated with cells at 37 °C, rinsed, lysed and measured for radioactivity. Binding quantified by tracing PACs labeled with a 5% fraction of ^{125}I -IgG relative to targeting Ab. Low biotin represents 2% biotinylated fraction included in the Pluronic PF127 and high biotin represents 5%. PAC-bound/cell calculated based on PAC concentration 2×10^{11} and cell density of $5 \times 10^4/cm^2$. Throughout the manuscript, data are shown as mean $\pm$ st dev; $n \geq 3$ unless otherwise stated. (B) Ab-PACs added 5000 PAC/cell incubated for 60 min at 37 °C, rinsed, permeabilized, fixed, and stained with Alexa 488 to particles (green) and DAPI to cell nuclei (blue). (C) Non-specific control IgG-PAC treated as in (B). (D–E) Endocytosis of PACs containing fluorescently labeled catalase with secondary red fluorescent staining to surface PAC Abs in non-permeabilized ECs. Yellow indicates surface-bound PACs whereas green shows endocytosed PACs. (D) Incubation 2500#/cell for 15 min; (E) incubation 2500#/cell for 30 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. *In vivo* endothelial targeting Ab-PAC. (A) Tissue distribution of intravenous injected PACs into mice after 30 min circulation time shown as injected dose per gram radioactivity relative to organ mass. Radioactivity of PAC traced by inclusion of 10% ^{125}I -labeled rlgG-SA per targeting Ab or IgG-control bound to PAC. $N = 5$. (B) Organ to blood ratio of % ID/g in selected organs. (C) Lung to liver ratio of Ab-PAC vs non-specific IgG PACs. Throughout this manuscript significance is shown as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.005$.

the airways. Analysis of the bronchoalveolar lavage fluid (BALF) obtained from LPS-challenged mice showed that PECAM-directed catalase PACKET, but not untargeted counterpart reduced pathological elevation of protein level in the alveolar compartment (Fig. 5B) and transmigration of leukocytes (Fig. 5C). Thus, a single injection of catalase PACKET alleviated pulmonary vascular damage and inflammation in this model of ALI, manifested by alveolar edema and leukocytosis.

3.4. Anti-inflammatory effects of PECAM-directed SOD PACKET

Next, we compared effects of catalase vs SOD delivered by PACKET on pathological endothelial activation by pro-inflammatory cytokine TNF. Our lab and others have documented that TNF induces endothelial expression of VCAM-1 via pathway involving superoxide production and NF- κ B activation [39]. Indeed, in this type of inflammatory/oxidative pathway, SOD, but not catalase PACKET, inhibited endothelial pathological activation (Fig. 6A). Of note, untargeted PACKET loaded with either AOE failed to affect VCAM-1 expression. This result indicates that even in a simplified model of cell culture allowing for a prolonged incubation with high doses of the formulations, specific delivery of SOD carrier to the cells is necessary for the effect. Based on this encouraging *in vitro* data, we appraised anti-inflammatory effect SOD PACKET in a mouse model of LPS-induced pulmonary inflammation described above. Analysis of the lung tissue homogenates revealed that PECAM-directed SOD PACKET, but not the untargeted IgG counterpart inhibited LPS-induced VCAM expression in the lung tissue (Fig. 6A, inset). Furthermore, PECAM-targeted SOD PACKET, but not the untargeted counterpart blocked elevation of the pulmonary level of MIP2 and TNF (Fig. 6B), cytokines formed in response to the primary LPS insult and implicated in propagation of inflammation [40].

4. Discussion

Therapeutic options for acute vascular oxidative stress and inflammation, which underlie the pathogenesis of many grave conditions, including acute lung injury (ALI), remain grossly sub-optimal. Optimizing vascular delivery may improve pharmacotherapy and clinical management of these conditions. For example, liposomal systemic delivery of clinically used antioxidants including N-acetyl cysteine (all of which failed to produce convincing clinical benefits) and targeted delivery to endothelial selectins of liposomes and other carriers loaded with clinically used anti-inflammatory drugs, alleviates indices of pathology in models of arthritis and systemic oxidative stress [41,42]. AOE catalase and SOD represent attractive biotherapeutics for this goal, given that

their problematic delivery to the therapeutic site is adequately resolved.

Design of targeted carriers for therapeutic enzymes is a challenging task. Enzymes encapsulated into protective carriers must be released to the therapeutic site, unless they metabolize small molecules that diffuse through the carrier, such as ROS. The latter paradigm ("protective cage-type" carriers) provides delivery platform for enzymes that exert their effects in the extracellular milieu and endocytic vesicles including lysosomes. Based on this, we encapsulated catalase and SOD into semi-permeable carriers based on biomaterials protecting AOE cargo from proteolytic degradation. In particular, controlled precipitation method employed in this work effectively encapsulates active AOE in carriers permeable to both H_2O_2 and superoxide, Protective Antioxidant Carriers (PAC). The magnetic responsiveness facilitates purification of PAC and can potentially extend the utility of PAC for targeted delivery in other clinical settings, including guidance to sites of stent deployment after angioplasty [43].

Enabling specific, ligand-mediated endothelial targeting of these carriers is the principal novelty of the present study. It was attained via conjugation of antibodies without affecting key physicochemical features of PAC. Such a conjugation on its own is not a trivial outcome. Biotin residues could alter distribution or balance of hydrophobic and hydrophilic components of the composition, leading to change in PAC assembly, size, AOE loading and stability. For example, inclusion of biotin beyond 4% alters the yield and morphology of polymeric carriers [44]. However, inclusion of biotinylated Pluronic and Ab/SA conjugation did not affect PAC, except endowing them with the avidity to the target molecule sufficient to achieve a highly effective and specific endothelial targeting.

By parameters of tissue uptake and specificity vs the untargeted counterpart, the pulmonary accumulation of PACKET exceeds the best reported in the literature [11,45,46]. In theory, the hydrophilic biotinylated polyethylene oxide moiety of Pluronic is likely to be oriented away from the particle to aqueous medium, thereby enhancing steric freedom of Abs attached to distal PEO ends and facilitating targeting [47]. In fact, the effectiveness of endothelial targeting of anti-PECAM PACKET is so high that it markedly inhibits the non-targeted uptake by RES. To our knowledge, an enhanced uptake in the desired site attained by affinity carriers is rarely accompanied by a significant reduction of hepatic uptake. In our case, however, targeting inhibits the hepatic uptake by ~30% (Fig. 4). Taking into account liver size, this is a substantial improvement of the biodistribution.

Targeting to PECAM enabled endosomal delivery of PACKET (Fig. 3D and E). This result is consistent with the extensive literature showing that endothelium effectively internalizes PECAM-targeted nanoparticles in cell culture and *in vivo* [5,27,28]. Endosomal

Fig. 5. Protection by endothelial-targeted catalase PACs from oxidative stress *in vitro* and *in vivo*. (A) Chromium 51-labeled endothelial cells incubated with catalase-loaded Ab or IgG PACs and exposed to H₂O₂ (10 mM). Protection is determined by reduction in ⁵¹Cr release as an indicator of cell death as compared to untreated cells exposed to H₂O₂ relative to naïve cells. (B) Brochoalveolar lavage (BAL) protein in lungs of mice exposed to LPS via intratracheal injection testing barrier function 24 h prior to intravenous injection of catalase loaded Ab vs IgG PACs. Lung lavaged 1 h after PAC administration. Protein measured by modified Lowry assay. (C) Reduction of white blood cell infiltration (same experiment as B). Cells counted 3× by light microscopy on a hemocytometer. Significance throughout manuscript shown as *p < 0.05, **p < 0.001, ***p < 0.005.

targeting is key for enabling quenching of endosomal superoxide anion by SOD PACKET, since this charged ROS diffuses poorly through biological membranes, unlike freely diffusible H₂O₂.

It is conventionally thought that in oxidative stress and inflammation, an excessive H₂O₂ flux causes damage to biomolecules leading to cell death and tissue injury, while superoxide mediates more subtle signaling mechanisms. In support of this notion, endothelial targeting of catalase alleviated tissue injury, while targeting SOD afforded little effects in animal models of severe acute oxidative stress [21,26] and vascular damage [48], typical of ischemia-reperfusion. However, in animal models of less severe vascular oxidative stress typical of hypertension and moderate inflammation, targeting of SOD alleviated endothelial dysfunction [21], cytokine-induced activation [39] and VEGF-induced permeability [15], while targeting catalase failed to provide these effects. Therefore, quenching of selected ROS is needed for inhibition of specific pathological mechanisms mediated by those molecules. In support of this paradigm, the present results show that targeted delivery of PACKET loaded with catalase vs SOD provides distinct and multifaceted protective effects both in cell culture and in animal models. Protective effects in a mouse model of acute LPS-induced lung inflammation provide the first evidence that vascular immunotargeting of AOE within PACKET affords therapeutic benefits unattainable by non-targeted counterparts. Keeping in mind differences between animal species and the challenge

conditions, LPS challenge is arguably one of the most widely used mouse models of severe pulmonary inflammation, which reflects some important characteristics of human acute lung injury [38,49].

The structure and size of AOE utilized in this study are remarkably different (MW 240 kD for tetramer catalase vs 32 kD for dimeric SOD), suggesting that this type of carriers may be employed for delivery of alternative therapeutic enzymes as well as other biotherapeutics [43]. Similarly, ROS substrates of these enzymes differ in charge and diffusivity. Therefore, PAC may provide a platform for delivery of enzymes that act via processing small-molecule substrates. Among other examples, such therapeutically relevant cargoes can include detoxifying enzymes decomposing endogenous or exogenous toxic compounds.

5. Conclusions

Herein we have provided evidence of strongly protective anti-inflammatory effects using a novel antioxidant enzyme nanocarrier formulation targeted to endothelial cells, obtained in an animal model of severe acute lung injury induced by endotoxin challenge. This approach enables endothelial delivery of active, protected from proteolysis catalase and SOD, providing specific protective antioxidant and anti-inflammatory effects in animal models of acute inflammation and/or oxidative stress. Due to the modular character of this drug delivery system, this platform has

Fig. 6. Reduction of inflammatory markers by Ab SOD PACs. (A) PECAM-targeted PACs loaded with SOD, but not catalase, reduce VCAM expression in activated endothelial cells. Western blot measurement of cell lysate for inflammatory-marker VCAM normalized to actin. ECs incubated 1 h with Ab/IgG PACs loaded with catalase or SOD, rinsed, and exposed to 10 ng/ml TNF at 37 °C for 4 h. Quantification of western blots measured by densitometry. Significance of Ab-SOD SOD demonstrated against each group. Inset: Mouse lung homogenate expression of VCAM measured by western blot from animals exposed to LPS for 24 h (as described in Fig. 5 B and C) injected with Ab/IgG PACs laden with SOD for 1 h. Red dotted lines in all graphs represent marker expression in treated (top) and naïve (bottom) controls. (B) PECAM-targeted PAC loaded with SOD reduce cytokine protein expression LPS exposed mice. Lung homogenate measured in animals injected with Ab or IgG PACs laden with SOD and exposed to LPS for 24 h for either MIP2 (left axis) or TNF (right axis) by ELISA. Significance measured against +LPS. Both concentration of MIP2/TNF determined relative to a standard curve where *R*² = 0.99. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

potential for site- and/or tissue-specific delivery of a variety of therapeutic cargoes to diverse targets.

Acknowledgments

EDH, CFG, and VRM acknowledge NIH grants R01 HL087036 and R01 HL073940. MC acknowledges.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2014.01.023>.

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