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Hemoglobin-based Oxygen Carriers Incorporating Nanozymes for the Depletion of Reactive Oxygen Species.

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KEYWORDS

Antioxidant activity; Blood substitutes; Cerium oxide nanoparticles; Hemoglobin; Layer-by-layer assembly; Nanozymes.

ABSTRACT

While transfusion of donor blood is a reasonably safe and well-established procedure, artificial oxygen carriers offer several advantages over blood transfusions. These benefits include compatibility with all blood types thus avoiding the need for cross matching, availability, lack of infection and long-term storage. Hemoglobin (Hb)-based oxygen carriers (HBOCs) are being explored as an “oxygen bridge” to replace or complement standard blood transfusions in extreme, life-threatening situations such as trauma in remote locations or austere battlefield or when blood is not an option due to compatibility issues or patient refusal due to religious objections.

Herein, a novel HBOC was prepared using the layer-by-layer technique. A poly(lactide-co-glycolide) core was fabricated and subsequently decorated with Hb and nanozymes. The Hb was coated with poly(dopamine) and preservation of the protein structure and functionality was demonstrated. Next, cerium oxide nanoparticles were incorporated as nanozymes and their ability to deplete reactive oxygen species (ROS) was shown. Finally, decorating the nanocarriers surface with poly(ethylene glycol) decreased protein adsorption and cell association/uptake. The as-prepared Hb-based oxygen nanocarriers were shown to be hemo- and biocompatible. Their catalytic potential was furthermore demonstrated in terms of superoxide radical- and peroxide-scavenging abilities, which were retained over multiple cycles. Overall, these results demonstrate that the reported nanocarriers show potential as novel oxygen delivery systems with prolonged catalytic activity against ROS.

1. INTRODUCTION

The transfusion of donor blood is a well-established and indispensable clinical procedure. Whole blood or isolated red blood cells (RBCs) are routinely employed to replenish blood loss during surgery and traumatic injury, and to treat diseases such as chronic anemia, hemophilia, and ischemia.¹⁻³ Unfortunately, blood transfusions come with limitations due to the need of typing and cross-matching, short storage life-time, limited supply, and the possible risks of viral and bacterial transmission.^{4,5} Therefore, research efforts have focused in developing RBC substitutes that circumvent the aforementioned drawbacks.

Hemoglobin (Hb)-based oxygen carriers (HBOCs) are one of the most prominent types of RBC substitutes being investigated, and they make use of the excellent oxygen (O_2)-carrying and delivering properties of native Hb.^{1,6} In HBOCs, Hb is chemically modified or encapsulated to overcome the adverse effects of intravascular cell-free Hb, which include renal toxicity, inflammation, and vasoconstriction.^{1,4,7} Multiple chemically-modified HBOCs underwent clinical development, but showed persistent safety concerns with increased risk of myocardial infarction and mortality.^{8,9} This toxicity was mainly attributed to the extravasation of the HBOCs. Because of their small size, the developed HBOCs could infiltrate in between the endothelial cells lining the capillary walls into the smooth muscle tissue. There, the HBOCs acted as nitric oxide (NO) scavengers. Being NO a potent vasodilator, its scavenging resulted in cardiovascular

complications. Therefore, Hb encapsulation strategies have gained attraction, shielding Hb from permeating through the blood vessels, while simultaneously providing a protective environment against tetramer dissociation and loss of function. Examples of encapsulation platforms developed so far include liposomes,¹⁰ polymeric nanoparticles,¹¹ hydrogels,¹² and metal-organic frameworks (MOFs).¹³ However, despite the advancements in recent years, fabrication of HBOCs that are non-toxic, hemocompatible, biodegradable, with good O₂ delivery properties, and are long-circulating in the bloodstream still remains a challenge.

In biological RBCs, Hb's O₂-delivery capacity is secured by an antioxidant system that involves both non-enzymatic (e.g., glutathione, ascorbic acid) and enzymatic (e.g., superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase) entities.¹⁴ This antioxidant system protects Hb from exogenous and endogenous reactive oxygen species (ROS). RBCs take up exogenous ROS when circulating in the microvasculature, since ROS are produced by the cellular components of the vascular wall.¹⁵ Additionally, endogenous ROS are produced within the RBCs following Hb's autoxidation into methemoglobin (metHb).^{14,16} MetHb conversion, apart from resulting in the loss of Hb's O₂-carrying ability, also leads to the production of the superoxide radical anion (O₂^{•-}). Within RBCs, metHb is reduced back to active Hb by cytochrome b5 reductase, while the formation of ROS is prevented. SOD catalyzes the dismutation of O₂^{•-} into hydrogen peroxide (H₂O₂).¹⁶ Next, H₂O₂ can be reduced by CAT into O₂ and water. However, in the absence of such an antioxidant system, H₂O₂ reacts with both metHb and oxygenated-Hb (oxy-Hb), initiating heme-mediated oxidative reactions.^{14,16}

Approaches to develop HBOCs where Hb is protected against oxidation, thus preserving its O₂-delivery properties, include the incorporation of CAT and SOD.^{17,18} Chang *et al.* pioneered this approach by co-polymerizing Hb together with CAT and SOD. The resulting polyHb-CAT-SOD complex was able to remove ROS while also decreasing metHb conversion and heme release.^{19,20} Despite these encouraging results, employing enzymes has several limitations which include high production costs, short catalytic half-lives, limited active sites, and environmental sensitivity.^{21,22} Therefore, herein, the novel approach of incorporating nanozymes is explored. Nanozymes are a type of nanoparticulate systems displaying enzyme-like properties, which overcome the aforementioned limitations.²³ Furthermore, since nanozymes are synthetic compounds, additional advantages include decreased batch-to-batch variations and lack of xenogeneic contamination.

While nanozymes offer enormous potential, a solitary example has considered them in the context of HBOCs. Hosaka *et al.* incorporated platinum nanoparticles (NPs) in their HBOC system, demonstrating high H_2O_2 and $\text{O}_2^{\cdot-}$ scavenging properties.²⁴ However, despite the promising results, no further studies were conducted in this direction.

From a different perspective, achieving long circulation times is a paramount requisite for the clinical translation of HBOCs. Upon intravenous administration, foreign materials are removed by the mononuclear phagocyte system (MPS).²⁵ To reduce recognition by the MPS, the adsorption of opsonins onto the NPs' surface has to be reduced or eliminated. The golden standard is decorating the surface with poly(ethylene glycol) (PEG), which creates steric hindrance and blocks protein-binding sites to reduce opsonisation and subsequent phagocytosis.²⁶

Thus, in this report, a novel HBOCs with antioxidant and stealth properties is presented (**Schematic 1**). In particular, the layer-by-layer (LbL) technique was employed to adsorb the different functional components onto a poly(lactide-*co*-glycolide) (PLGA) core. Since PLGA is a biodegradable, biocompatible, and FDA approved polymer, PLGA-NPs are being widely explored as colloidal carrier systems. Cerium oxide (CeO_2)-NPs, which are a new class of nanozymes with excellent ROS-scavenging activity, were selected as the antioxidant component. The assembly was finally capped with a poly(*L*-Lysine)-*graft*-PEG (PLL-*g*-PEG) co-polymer to obtain a low-fouling effect. The O_2 carrying potential, catalytic activity, and the stealth-like properties of this new HBOC, as well as the overall biocompatibility in terms of hemolytic rate and cell viability, were evaluated. By characterizing the multifaceted properties of the system, we hope to demonstrate the potential of these novel HBOCs.

2. MATERIALS AND METHODS

2.1. Materials.

Tris(hydroxymethyl)aminomethane (TRIS), phosphate buffer saline (PBS), *tert*-butanol, dopamine (DA), Amicon Ultra-15 Centrifugal Filter Unit (10 kDa MWCO), cerium(III) nitrate hexahydrate ($\text{Ce}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$), citric acid, hydrochloric acid (HCl), nitric acid (HNO_3), ammonia (25% solution), , hydrogen peroxide (H_2O_2), horseradish peroxidase (HRP), CAT from bovine liver, SOD from bovine erythrocytes, cell proliferation reagent WST-1, ethylenediaminetetraacetic

acid solution (EDTA), xanthine, xanthine oxidase (XO) from bovine milk, potassium dioxide (KO_2), Poly(*L*-lysine) (PLL, M_w 30-70 kDa), fluorescein isothiocyanate (FITC)-labelled immunoglobulin G (IgG^F) from human serum, Dulbecco's Modified Eagle's Medium-high glucose (DMEM D5796), penicillin/streptomycin, fetal bovine serum (FBS), trypsin, and the human umbilical vein endothelial cell line (HUVEC) were purchased from Merck Life Science A/S (Søborg, DK). AmplexTM Red reagent and PrestoBlue cell viability reagent were purchased from ThermoFisher Scientific (Waltham, MA, USA). Bovine blood with citrate (Product no. 77667) was purchased from SSI Diagnostica A/S (Hillerød, DK). Hb was obtained by extraction from the bovine blood (see ESI). Endothelial Cell Medium (ECM) Kit supplemented with 5% FBS, 1% penicillin/streptomycin solution, and 1% endothelial cell growth supplement was purchased from Innoprot (Derio-Biskaia, ESP). The RAW 264.7 mouse macrophage cell line was purchased from European Collection of Authenticated Culture Collections (ECACC, Wiltshire, UK).

PLGA-NPs, FITC-labelled bovine serum albumin (BSA) (BSA^F) and PLL-*g*-PEG were synthesized (see ESI).

TRIS 1 contains 10 mM TRIS (pH 8.5); TRIS 2 contains 10 mM TRIS and 150 mM NaCl (pH 7.4). All buffers were made with ultrapure water (Milli-Q (MQ), gradient A 10 system, TOC < 4 ppb, resistance 18 MV cm, EMD Millipore, USA).

2.2. Cell Culture.

RAW 264.7 cells were cultured in DMEM supplemented with FBS (10% v/v) and penicillin/streptomycin (1% v/v, 10 000 U mL^{-1} and 10 $\mu\text{g mL}^{-1}$, respectively). HUVECs were cultured in ECM supplemented with FBS (5% v/v), penicillin/streptomycin (1% v/v, 10 000 U mL^{-1} and 10 $\mu\text{g mL}^{-1}$, respectively), and endothelial cell growth supplements (1% v/v). Both cell lines were cultured at 37 °C in a humidified incubator with 5% CO_2 .

2.3. Fabrication and Characterization of Polydopamine (PDA)-Coated Hb (Hb^{PDA}).

The coating of Hb with PDA was based on the oxidative polymerization of DA in TRIS 1.²⁷ Briefly, solutions of Hb (8 mg mL^{-1}) and DA (1.6 mg mL^{-1}) in TRIS 1 were mixed at a 1:1 volume ratio and transferred to a tube rotator for 3 h. The resulting Hb^{PDA} was washed in TRIS 2 (3×, 9700

g, 5 min) using a bench-top centrifuge (MiniSpin, Eppendorf AG, Hamburg, DE) and Amicon centrifugal filters.

The hydrodynamic size of Hb^{PDA} was evaluated by dynamic light scattering (DLS) (Zetasizer nanoseries nano-ZS, Malvern Panalytical Ltd, Malvern, UK). To determine the secondary structure, Fourier-transform infrared spectroscopy (FTIR) analysis was conducted using a spectrometer Spectrum 100 (Perkin Elmer Inc., Wellesley, MA, USA). Circular dichroism (CD) spectra were obtained using a Jasco J-815 spectropolarimeter (JASCO, Essex, UK). To assess the Hb's functionality, a gas binding and release study was conducted using a UV-2600 UV-Vis Spectrophotometer (Shimadzu Corp., Kyoto, JP). The oxygen dissociation curves (ODC) were obtained using a Hemox analyzer (TSC Scientific Corp., New Hope, PA, USA).

2.4. Fabrication and Characterization of CeO₂-NPs.

2.4.1. Fabrication and characterization of CeO₂-NPs. Citrate-capped CeO₂-NPs were synthesized following a reported protocol.²⁸ Specifically, citric acid (120 mg) was added to a Ce(NO₃)₃ • 6H₂O solution (12.5 mL, 21.71 mg mL⁻¹ in MQ) followed by gentle mixing, poured rapidly into an ammonia solution (50 mL, 3 M) and stirred for 2 h. The obtained CeO₂-NPs were purified by adding HCl (~25 mL, 5 M) until precipitation occurred. The CeO₂-NPs were allowed to sediment, the supernatant was then removed, and the CeO₂-NPs were re-suspended in MQ. These washing steps were repeated three times. After the final wash, the supernatant was removed and the pH of the CeO₂-NPs suspension was adjusted to neutral pH using an ammonia solution (1 M). All glassware was treated with Aqua Regia (HNO₃:HCl, 1:3 molar ratio) before usage.

The cerium content of the CeO₂-NPs suspension was determined by inductively coupled plasma mass spectrometry (ICP-MS) (iCAPQ, ThermoFisher Scientific, Waltham, USA). The content was also characterized by UV-Vis spectroscopy (NanoDrop 2000c, ThermoFisher Scientific, Waltham, USA) (**Figure S1**). The hydrodynamic size, polydispersity (PDI), and zeta (ζ)-potential of CeO₂-NPs were evaluated using the Zetasizer. The size, morphology, and selected area diffraction (SAD) patterns of the CeO₂-NPs were evaluated using a Tecnai T20 G2 transmission electron microscope (TEM) (Thermo Fisher Scientific, Waltham, MA, USA).

2.4.2. Catalytic Activity of CeO₂-NPs. To determine the SOD-like activity (O₂^{•-} scavenging), the WST-1 assay was employed. XO (10 µL, 0.05 U mL⁻¹) was added to 200 µL of reagent mixture containing WST-1 (2.5% v/v), EDTA (0.1 mM), and xanthine (0.1 mM), mixed with CeO₂-NPs (100 µL, 0 – 2 mM) or SOD (100 µL, 0 – 1000 U mL⁻¹), all in TRIS 2. The mixture was incubated for 15 min using a thermoshaker (PHMT Thermoshaker, Grant-bio, UK) at 37 °C with a shaking speed of 1400 rpm. Next, 180 µL of the suspension were transferred to a 96-well plate and the absorbance (Abs) at 438 nm was measured using a multimode plate reader (Tecan Spark, Tecan Group Ltd., Männedorf, CH). TRIS 2 (100 µL) with 200 µL of reagent mixture, with and without XO (10 µL, 0.05 U mL⁻¹) were used as positive and negative controls, respectively, to calculate the normalized Abs (nAbs).

The CAT-like activity (H₂O₂ scavenging) was assessed using the Amplex Red assay. H₂O₂ (10 µL, 0.09 mM) was added to CeO₂-NPs (100 µL, 0 – 5 mM) or CAT (100 µL, 0 – 14 000 U L⁻¹), all in TRIS 2, and incubated for 15 min in the thermoshaker. HRP (100 µL, 2 U mL⁻¹) and Amplex Red (10 µL, 0.1 mM) were added to the suspension, followed by 5 min incubation in the thermoshaker. 180 µL of the suspension were transferred to a 96-well plate and the fluorescence intensity (FI) of the resorufin product was measured using the plate reader (λ_{ex}/λ_{em}: 530/586 nm). TRIS 2 (100 µL) incubated with and without H₂O₂ (10 µL, 0.09 mM) were used as positive and negative controls, respectively, to calculate the normalized FI (nFI).

2.5. Fabrication and Characterization of the Hb-loaded Nanocarriers (NCs).

The LbL technique was used for the assembly of the Hb-loaded NCs. PLGA-NPs (1000 µg) were incubated in DA solutions (300 µL, 0.5 mg mL⁻¹ in TRIS 1) for 30 min. The PDA-coated PLGA-NPs were washed in TRIS 2 (2×, 4847 g, 4 min). Next, the following layers in TRIS 1 were deposited for 10 min: PLL (300 µL, 1.0 mg mL⁻¹), Hb^{PDA} (300 µL, 2.0 mg mL⁻¹), PLL (300 µL, 1.0 mg mL⁻¹), CeO₂-NPs (300 µL, 3.75 mM), and PLL-g-PEG solutions (300 µL, 1.5 mg mL⁻¹). After each deposition step, at least 2 washes were conducted. The concentrations were determined by ζ-potential saturation curves (**Figure S2**).

The hydrodynamic size, PDI, and ζ-potentials were measured using the Zetasizer. Encapsulation content was determined by the Nanodrop and ICP-MS. CryoTEM imaging was performed using a FEI Tecnai G2 20 TWIN TEM. The final PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs were visualized by

differential interference contrast (DIC) microscopy (Olympus Inverted IX83 microscope equipped with a 60× oil-immersion objective).

2.6. Evaluation of the Stealth Coating.

2.6.1. Protein Adsorption Study. The PEGylation effect of PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs was assessed by a protein adsorption. BSA^F (100 μL, 1.0 mg mL⁻¹) or IgG^F (100 μL, 1.0 mg mL⁻¹) in TRIS 2 was added to a suspension of either PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs or PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs (100 μL, $\sim 2.7 \times 10^6$ NCs in TRIS 2) and incubated for 4 h in the thermoshaker. Next, the samples were washed in TRIS 2 (2×, 6705 g, 4 min) to remove unbound proteins. The FI of the nanocarriers resulting from the adsorbed BSA^F and IgG^F onto the NCs surface was evaluated by a BD Accuri C6 flow cytometer (BD Biosciences, Sparks, MD, USA).

2.6.2. Cell Association Study. In a 96-well plate, 30 000 RAW 464.7 or 15 000 HUVEC cells per well were seeded and allowed to attach for 24 h. The cells were then washed in PBS (2×, 200 μL) and incubated with different amounts of PLGA^F/Hb^{PDA}/(CeO₂-NPs)-NCs or PLGA^F/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs (200 μL, 0 – 7.5×10^6 NCs mL⁻¹ in cell media) for 4 h. After incubation, the cells were washed in PBS (3×, 200 μL) and detached from the wells using trypsin (60 μL, 0.5 mg mL⁻¹). The cell suspensions were collected by adding FBS-free media (200 μL per well) and the cell association/uptake was evaluated by flow cytometry. The cell mean fluorescence intensity (CMFI) and cellular uptake efficiency (CUE) were assessed. Cells without exposure to the NCs were used as controls. The CUE was determined as the percentage of cells with a CMFI higher than the autofluorescence level of the cells. The CMFI was normalized (nCMFI) to PLGA^F/Hb^{PDA}/(CeO₂-NPs)-NCs at 7.5×10^6 NCs mL⁻¹.

2.7. Biocompatibility.

2.7.1. Cell Viability. In a 96-well plate, 30 000 RAW 464.7 or 15 000 HUVEC cells per well were seeded and allowed to attach for 24 h. Next, the cells were washed in PBS (2×, 200 μL) and incubated with different amounts of PLGA^F/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs (200 μL, 0 – 3.0×10^7 NCs mL⁻¹ in cell media). After 4 h incubation, the cells were washed in PBS (3×, 200 μL) and PrestoBlue (100 μL, 10% v/v in cell media) was added. The cells were incubated for 1 h and then

the supernatants were transferred to a 96-well plate to measure the resazurin product ($\lambda_{\text{ex}}/\lambda_{\text{em}}$: 535/615 nm). Cells only were used as the positive control, and media only was used as the negative control to calculate the normalized cell viability (nCV).

2.7.2. Hemocompatibility. The hemocompatibility of both PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs and PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs was investigated by a haemolysis rate test. Whole blood from healthy donors was collected in heparin-coated tubes and washed in PBS (3×, 1000 g, 15 min, 4 °C). The cell-containing pellet was re-suspended in PBS (50 mL), and 200 µL of this diluted blood cell suspension at 37 °C was added to the NCs suspensions (300 µL, 0 – 1.2 ×10⁷ NCs in PBS). The mixtures were incubated for 2 h in the thermoshaker (with gentle shaking). As negative and positive controls, the diluted blood cell suspension (200 µL) was incubated in PBS or MQ water (300 µL), respectively. Next, the samples were spun down (1000 g, 10 min) and the supernatants were collected followed by an additional centrifugation step (13 000 g, 10 min). The new supernatants were transferred to a 96-well plate and the Abs at 540 nm was recorded. The haemolysis rate was calculated as: haemolysis rate (%) = (experimental value – negative control)/(positive control – negative control) × 100. In parallel, the remaining cell-containing pellet after the first centrifugation step was collected and re-suspended in PBS. The morphology of the blood cells was assessed using the DIC microscope.

2.8. Catalytic Activity.

2.8.1. SOD-like activity. The influence of each functional layer of the Hb-loaded NCs on the decomposition of O₂^{•-} was assessed using the WST-1 assay. Specifically, PLGA^F-NPs, PLGA^F/PDA-NCs, PLGA^F/Hb^{PDA}-NCs, PLGA^F/Hb^{PDA}/(CeO₂-NPs)-NCs and PLGA^F/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs were investigated. XO (10 µL, 0.05 U mL⁻¹) was added to 200 µL of reagent mixture containing WST-1 (2.5% v/v), EDTA (0.1 mM), and xanthine (0.1 mM), mixed with a NC suspension (200 µL, 1.5 ×10⁶ NCs), all in TRIS 2. The suspensions were incubated for 15, 30, and 60 min using the thermoshaker, after which the NCs were spun down (6705 g, 4 min), 180 µL of the supernatant was transferred to a 96-well plate, and the Abs at 438 nm was measured. TRIS 2 (100 µL) mixed with 200 µL of reagent mixture, with and without XO (10 µL, 0.05 U mL⁻¹) were used as positive and negative controls, respectively, to calculate the nAbs.

2.8.2. Combined SOD- and CAT-like activity. The ability of the Hb-loaded NCs to catalyze the reduction of $O_2^{\cdot -}$ into O_2 and H_2O_2 , followed by the subsequent decomposition of H_2O_2 into O_2 and H_2O , was assessed by the Amplex Red assay.²⁷ Specifically, PLGA^F-NPs, PLGA^F/PDA-NCs, PLGA^F/Hb^{PDA}-NCs, PLGA^F/Hb^{PDA}/(CeO₂-NPs)-NCs, and PLGA^F/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs were investigated. KO₂ (10 μ L, 0.14 mM in TRIS 2) was added to the NCs suspensions (200 μ L, 1.5×10^6 NCs in TRIS 2) and incubated for 15, 30, and 60 min in the thermoshaker. Next, the NCs were spun down (6705 g, 4 min) and 180 μ L supernatant was collected and incubated with a solution of HRP (100 μ L, 2 U mL⁻¹ in TRIS 2) and Amplex Red (10 μ L, 0.1 mM in TRIS 2) for 5 min. Finally, 180 μ L of the suspension was transferred to a 96-well plate and the FI ($\lambda_{ex}/\lambda_{em}$: 530/586 nm) was measured. TRIS 2 (200 μ L) incubated with and without KO₂ (10 μ L) were used as positive and negative controls, respectively, to calculate the nFI.

2.8.3. Multiple Cycles of SOD- and CAT-like Activity. The PLGA^F/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs (200 μ L, 1.5×10^6 NCs in TRIS 2) were allowed to interact with KO₂ (10 μ L, 0.14 mM) for 15 min using the thermoshaker after which the supernatant was collected and incubated with a solution of HRP (100 μ L, 2 U mL⁻¹) and Amplex Red (10 μ L, 0.1 mM) for 5 min in the thermoshaker. Finally, 180 μ L of the suspension was transferred to a 96-well plate and the FI ($\lambda_{ex}/\lambda_{em}$: 530/586 nm) was measured. Next, the PLGA^F/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs were re-suspended in fresh TRIS 2 (200 μ L) and incubated again in a KO₂ solution (10 μ L, 0.14 mM) to conduct the second cycle of the cascade activity. The aforementioned process was repeated for five subsequent cycles. The cascade activity was normalized to TRIS 2 (200 μ L) incubated with KO₂ (10 μ L, 0.14 mM).

2.9. Statistical Analysis.

The statistical difference between different conditions was evaluated using GraphPad Prism 8 software. A one-way ANOVA with a confidence level of 95% ($\alpha = 0.05$) using Tukey's multiple comparisons test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$) was employed.

3. RESULTS AND DISCUSSION

3.1. Fabrication of the Hb-loaded NCs.

3.1.1. Fabrication and Characterization of Hb^{PDA}.

Despite its excellent O₂ binding and releasing properties, Hb molecules break down into dimers and monomers when administered in their free form, causing severe toxicity.^{1,7} Therefore, to avoid Hb's dissociation, we introduce a PDA coating. DA, under mild basic conditions (TRIS 1), has the ability to self-polymerize into PDA onto virtually any type of substrate independent of shape and material.^{29–31} Importantly, unlike other Hb stabilization methods (e.g., using intra- and inter-molecular modifications),^{1,4} PDA coatings closely bond onto Hb's surface with barely any chemical reaction with its functional groups. Additionally, we, and others, have demonstrated that PDA has ROS scavenging abilities.^{27,32} The incorporation of PDA has been previously employed to minimize the oxidation of HBOCs. Specifically, individual Hb molecules have been PDA-coated, but also larger particles fully made of Hb.^{33–36}

Herein, Hb's coating by PDA was conducted employing a weight ratio 5:1 of Hb and DA for 3 h. **Figure 1A** shows how the colour of the suspension changed over time from red to dark brown, thereby demonstrating the DA polymerization into PDA. The size of the Hb^{PDA}, as evaluated by DLS, showed a slight increase from 5.2 nm (for uncoated Hb) to 7.0 nm (for Hb^{PDA}) (Figure 1B). The size distribution furthermore indicates individual PDA-coated Hb molecules instead of Hb^{PDA} aggregates. This is in agreement with Wang *et al.*, which showed a similar hydrodynamic size for PDA-coated Hb, obtaining a ~1-2 nm size increase after coating, and using the same PDA polymerization conditions.³⁴

Proteins secondary structure is central to their function, and preservation of this structure is a strong indication for preserved protein activity. Thus, FTIR spectroscopy was used to evaluate Hb's functional regions following PDA coating (Figure 1C). The comparison of FTIR spectra of Hb and Hb^{PDA} shows how both spectra display almost identical bands at 1642 cm⁻¹ (amide I) and 1530 cm⁻¹ (amide II). Preservation of wavenumbers or band ratios for both amide I (corresponding to C=O stretching) and amide II (corresponding to N-H bending and C-N stretching) indicates that Hb's chemical structure is well maintained following PDA coating. The bands of the PDA spectrum at 875 cm⁻¹ and 809 cm⁻¹ correspond to the three and the five substituted benzene stretching vibrations, respectively. While previous reports have shown increase in adsorption for the bands at 875 cm⁻¹ and 809 cm⁻¹ following PDA coating,³³ the two bands are not present in our Hb^{PDA} spectrum. We attribute the absence of these bands to the deposition of a thinner PDA coating due to the usage of a lower DA concentration. Hb's secondary structure following coating with PDA

was further investigated by CD in the range of 197 to 260 nm (Figure 1D). The CD spectrum of Hb^{PDA} is almost identical to the spectrum of Hb. Both spectra show two absorption bands at 209 and 220 nm, which are characteristic for α -helical structures.³⁷ Such a similar helicity for the PDA-coated Hb indicates preservation of Hb's secondary structure. As control, Hb heated to 60 °C (as a model of partially denatured Hb) and Hb heated to 100 °C (as a model of fully denatured Hb) were considered. Here, it can be observed denaturation leads to a large dip in intensity and, eventually, complete loss of the of the α -helix structure.

The effect of the PDA coating on Hb's native bioactivity was also investigated by monitoring the ability of Hb^{PDA} to reversibly bind and release O₂. **Figure 2A** shows how the initial Hb^{PDA} (O₂ - cycle 1) displays the characteristic adsorption bands of oxy-Hb. Specifically, a main band at 409 nm (Soret peak) and two additional bands in the visible region (at 539 nm and 575 nm) can be observed. Next, following purging with nitrogen gas (N₂ – cycle 1), the Soret band shifted to 427 nm, and a single band appeared at 553 nm, indicating the presence of deoxygenated Hb. Following on, the deoxygenated Hb^{PDA} was purged with compressed air, resulting in the appearance of the oxy-Hb absorbance bands and demonstrating the ability of Hb^{PDA} to reversibly bind and release O₂. After 3 weeks of storage at 4 °C, the Hb^{PDA} samples still showed this reversibility (**Figure S3**). A second N₂ cycle and a third O₂ cycle were also conducted, and the changes in the UV-Vis spectra highlighting the ability of Hb^{PDA} to bind and release O₂ over multiple rounds. Exposure to carbon monoxide (CO) gas was also evaluated. A shift in the Soret band to 417 nm together with the shift of the two visible-bands to 537 nm and 568 nm, demonstrate the conversion of oxy-Hb to CO-Hb.

To get a deeper insight on the O₂ binding abilities of Hb^{PDA}, the ODC of Hb^{PDA} was assessed. Figure 2B shows the dependency of Hb's saturation with O₂ on the pO₂ with a sigmoidal shape, indicating cooperative O₂ binding for both free Hb and the PDA-coated Hb^{PDA}. The oxygen equilibrium parameter p50, which is the pO₂ at which 50% O₂ saturation is achieved, was 20.9 mmHg for free Hb. A decrease in p50 to 15.3 mmHg was observed following PDA coating, which indicates increased O₂ affinity for Hb^{PDA}. It could be hypothesized that the PDA coating restricts Hb's movement from the relaxed and oxygenated state into the tense and deoxygenated one. This could subsequently result in O₂ release at a lower pO₂ as compared to free Hb. Achieving low p50 is beneficial when developing HBOCs since it minimizes premature O₂ release in the precapillary arterioles, thus maximizing O₂ supply in hypoxic tissues. The Hill coefficient (*n*), calculated as 1.2

(and 2.1 for free Hb) is similar to previously reported n -values for HBOCs.^{38,39} Although there is a decrease in n following PDA coating, cooperativity is still retained ($n > 1$). The cooperativity is a result of subunit-subunit interaction,⁴⁰ thereby indicating that the tetrameric Hb structure was not degraded into monomers after PDA coating. Thus, O₂ binding to one of the heme groups of Hb^{PDA}, results in increased O₂ affinity for the remaining free hemes, facilitating maximum O₂ loading. In turn, O₂ release decreases the O₂ binding affinity of the remaining ligated hemes, thus facilitating complete O₂ release.

3.1.2. Fabrication and Characterization of CeO₂-NPs.

Over time, Hb auto-oxidizes into methHb which results in impaired O₂ binding and releasing properties and oxidative side reactions mediated by the heme group.¹⁴ Within biological RBCs, Hb conversion into methHb is prevented by a multi-faceted antioxidant system. Our approach however is to employ nanozymes, a novel type of nanoparticulate systems displaying enzyme-like properties but overcoming the limitations of biological enzymes such as short half-life, environmental sensitivity, and high production costs.^{21,23} Additionally, nanozymes exhibit better recyclability over their natural counterparts, which is a crucial aspect if they are expected to prevent methHb conversion over an extended period of time. In particular, CeO₂-NPs were chosen as antioxidant nanozymes. Within CeO₂-NPs, Ce(III) and Ce(IV) oxidative states co-exist, producing a redox couple.⁴¹ This interchange between valence states of the Ce atoms makes it possible for the CeO₂-NPs to react catalytically with H₂O₂ and O₂^{•-}, thereby replacing the functions that are normally performed by CAT and SOD, respectively.

Thus, CeO₂-NPs were synthesized and characterized for their catalytic activity. The as-prepared CeO₂-NPs displayed a hydrodynamic diameter of 2.6 ± 0.5 nm, a 0.568 PDI, and a ζ -potential of -26 ± 7 mV (**Figure 3A**). TEM imaging showed that the CeO₂-NPs were monodisperse with an average size of 2.2 nm (Figure 3B), which is in agreement with the size obtained using DLS. The crystalline nature of the CeO₂-NP was directly observable in Figure 3C, showing a phase contrast image of an individual CeO₂-NP oriented along the $[1\bar{1}0]$ zone axis. The (111) interplaner spacing measured 0.31 nm corresponding to CeO₂. TEM-SAD was performed to further confirm the crystallinity of the CeO₂-NPs. A polycrystalline SAD pattern (Figure 3D) was obtained with

diffraction rings corresponding to the $Fm\bar{3}m$ space group with lattice parameter $a = 0.54$ nm, which matches well with CeO_2 's fluorite crystal structure ($Fm\bar{3}m$, $a = 0.54$ nm).⁴²

Next, the antioxidant activity of the CeO_2 -NPs was assessed. First the SOD-like activity, measured by the ability of the CeO_2 -NPs to scavenge $O_2^{\cdot-}$ over time, was evaluated using the WST-1 assay and compared to the natural enzyme (**Figure 4A**). Within this assay, O_2 is converted into $O_2^{\cdot-}$ by the xanthine/ XO system (Figure 4Ai). $O_2^{\cdot-}$ oxidizes the WST-1 reagent into formazan which can be detected by UV-Vis at 450 nm. However, SOD-like activity of the CeO_2 -NPs or native SOD converts the $O_2^{\cdot-}$ into O_2 and H_2O_2 , thereby decreasing the amount of $O_2^{\cdot-}$ present in the system, and thus subsequently decreasing the amount of oxidized formazan being produced. An increase in both CeO_2 -NPs and SOD concentrations results in an enhanced $O_2^{\cdot-}$ removal, as shown by the decreased nAbs values (Figure 4Aii). Almost complete $O_2^{\cdot-}$ depletion is achieved since a maximum reduction to 7% nAbs is observed for concentrations of 1 mM CeO_2 -NPs and 50 U mL⁻¹ SOD. Additionally, the SOD-like activity of CeO_2 -NPs stored in solution at 4 °C was shown to be maintained for at least over a period of 21 days (Figure 4Aiii).

The CAT-like activity of the CeO_2 -NPs was investigated by measuring their H_2O_2 scavenging ability using the Amplex Red assay and compared to that of the biological enzyme (Figure 4B). Within this assay, H_2O_2 acts as a co-substrate for the conversion of the Amplex Red reagent, in presence of HRP, into the fluorescent resorufin product (Figure 4Bi). CAT-like activity of the CeO_2 -NPs or native CAT converts H_2O_2 into H_2O and O_2 , thereby decreasing the amount of H_2O_2 present in the system, and subsequently decreasing the amount of resorufin product being produced. Figure 4Bii shows that increasing concentrations of both CeO_2 -NPs and CAT result in decreased H_2O_2 amounts, as indicated with the lower nFI values. While, for CeO_2 -NPs, plateau values of ~ 17% in nFI were obtained for a concentration 0.5 mM; complete H_2O_2 depletion was detected for a concentration of 12 U mL⁻¹ CAT. Although, in this case, complete H_2O_2 depletion was obtained only with the natural enzyme, the H_2O_2 scavenging ability of CeO_2 -NPs remained almost unchanged for a period of at least 21 days when stored in solution at 4 °C (Figure 4Biii). In contrast, the CAT enzyme experiences a complete loss of catalytic activity within 20 days when stored at 4 °C.⁴³ Therefore, the long-term stability of the antioxidant properties of CeO_2 -NPs makes them particularly interesting for the fabrication of HBOCs with long shelf life.

3.1.3. Fabrication and Characterization of the Hb-loaded NCs.

Different HBOCs encapsulation strategies have been developed, including lipidic,¹⁰ polymeric,¹¹ and MOF-based platforms. MOFs feature well-defined pore structures which allow for high Hb encapsulation.¹³ Interestingly, zinc-based MOFs were shown to protect the encapsulated Hb against oxidation by H₂O₂.⁴⁴ Hb has been also encapsulated within lipidic vesicles, also known as liposomes. Such a system has already been widely evaluated in pre-clinical models showing circulation lives up to 65.2 h and up to 72 h in rabbits and monkeys, respectively.^{45,46} Herein, however, the polymeric platform is chosen. Polymeric NPs are more flexible in encapsulating different entities, due to their high tunability and choice in materials. Specifically, the LbL technique, which involves the alternating deposition of interacting entities (e.g., polymers but also NPs or liposomes) onto a core allows for these complex systems.^{47–49} Thus, the LbL technique was employed to incorporate the Hb^{PDA} and CeO₂-NPs onto PLGA-NPs.

Negatively charged PLGA-NPs with a ζ -potential of -19 mV, an average hydrodynamic size of 217 nm, and a 0.042 PDI were synthesized. However, their negative charge does not allow for the adsorption of Hb^{PDA} and CeO₂-NPs, which are also negatively charged. Thus, a PDA layer which will shield the environment from the organic core while also facilitating surface modification,⁵⁰ is first deposited (PLGA/PDA-NCs). An increase in ζ -potential (to -15 mV) and the dark appearance of the PLGA/PDA-NCs, as shown in the inset, confirmed the successful deposition of PDA (**Figure 5A**). Next, in order to achieve charge reversal to allow for the deposition of the negatively charged Hb^{PDA} and CeO₂-NPs, PLL intermediate layers were deposited. PLL was chosen since, apart from being a positively charged and biocompatible polymer, it allows for Michael-type addition and Schiff-base formation with the catechol moieties of the PDA layer, thus creating strong chemical bonding between the layers.⁵⁰ As shown in Figure 5A, the ζ -potential became positive (+14 mV) after PLL deposition. Next, the Hb^{PDA} layer resulted in PLGA/Hb^{PDA}-NCs with a ζ -potential of -16 mV, indicating a successful adsorption. PLL was used again as a positively charged separation layer (+10 mV), followed by the deposition of CeO₂-NPs which rendered a negative ζ -potential of -19 mV (PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs). Finally, to obtain Hb-loaded NCs with a stealth coating, the positively charged copolymer PLL-g-PEG was deposited onto the NCs yielding PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs with a final overall charge of +9 mV. The loading of Hb and CeO₂-NPs was quantified by ICP-MS. Specifically, $150.3 \pm 26.7 \mu\text{g}$ Hb had

been entrapped within a NC as shown by measuring the iron content. $0.213 \pm 0.007 \mu\text{mol CeO}_2\text{-NPs}$ were incorporated per NC as shown by measuring the cerium content by ICP-MS.

The final PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs had an average hydrodynamic diameter of 269 nm, with a 0.055 PDI. DIC imaging furthermore showed a monodisperse suspension (Figure 5Bi) while cryo-TEM imaging confirmed the presence of the deposited CeO₂-NPs (Figure 5Bii), which are presented as dots on the surface of the nanocarrier (as indicated by the black arrows). While the CeO₂-NPs were mainly deposited in a monodisperse way, bigger CeO₂-NPs aggregates on top of the NCs could also be observed (open arrow). We suggest that aggregation could be due to the presence of NaCl in the final TRIS 2 where the NCs are suspended, which has been previously reported to have an aggregation effect onto citrate-stabilized particles.⁵¹

3.2. Evaluation of the Stealth Coating.

To improve the circulation time of nanoparticulate systems, surface PEGylation is a well-established strategy. PEGylation prevents opsonisation by blocking protein-binding sites and/or creating steric hindrance due to its flexible hydrophilic polymer chains. In this study, PEGylation was easily achieved by coating the LbL-assembled nanocarrier with PLL-g-PEG. This comb-like copolymer was chosen to provide sufficient electrostatic binding sites between PLL backbone and the PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs, while the non-ionic hydrophilic PEG chains extend outwards, providing antifouling properties.⁵²

To assess the effect of PEGylation as stealth coating, a protein adsorption study was performed. Uncoated PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs and PEGylated PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs were incubated in albumin and immunoglobulin solutions (BSA^F and IgG^F, respectively), which are the most abundant proteins in blood.²⁷ The results show a different PEGylation effect depending on the studied protein (**Figure 6A**). In particular, the absorption of BSA^F onto the NCs is very limited independently of the end coating. As such, both PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs and PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs experience only a 15% increase in nFI as compared to the controls (NCs without protein exposure). While we attribute the low adsorption onto PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs to the stealth properties of the PEG coating, previous results have shown little to non-significant BSA adsorption for negatively charged CeO₂-NPs, which has been attributed to their electrostatic repulsion.⁵³ In contrast, for IgG^F, PEGylation has a tremendous

impact with similar nFI readings for the PEGylated NCs (11%) and the control (9%). In contrast, a 91% increase in nFI is observed for the uncoated NCs. As shown by Zhang *et al.*, IgG type proteins have the ability to destroy the citrate ligands of citrated-capped inorganic NPs, subsequently binding onto the inorganic core.⁵⁴ Furthermore, during the destruction of the citrate ligands, the inorganic NPs cores are temporarily exposed, which can lead to the irreversible aggregation of the NPs as a result of the strong van der Waals interactions.⁵⁴ This aggregation will then translate into accelerated clearance by the MPS.

The effect of PEGylation was also assessed *in vitro* by means of cell association studies. Two model cell lines, RAW 264.7 macrophages and HUVEC endothelial cell lines were used. Macrophages are the first line of defense against intruding pathogens, while endothelial cells line the inside of the blood vessels. The cells were exposed to uncoated PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs and PEGylated PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs, and the CUE and CMFI of the cells incubated for 4 h with different amounts of NCs, were evaluated. The CUE shows a slight increase upon incrementing the amount of NCs for both cell lines and for both uncoated and PEGylated NCs (Figure 6B). Importantly, for both cell lines, there is a significant decrease in CUE following incubation with the PEGylated NCs as compared to the uncoated counterparts. Such a decrease in CUE ranges from 23% (for HUVEC cells exposed to 7.5×10^6 NCs mL⁻¹) to up to 46% (for RAW 264.7 cells exposed to 1.5×10^6 NCs mL⁻¹). The nCMFI readings are in agreement with the CUE results (Figure 6C), showing a decrease in nCMFI when both cell lines are incubated with the PEGylated NCs. This decrease in nCMFI ranges from 8% (for HUVEC cells exposed to 7.5×10^6 NCs mL⁻¹) to up to 48% (for RAW 264.7 cells exposed to 7.5×10^6 NCs mL⁻¹). Thus, functionalization with PLL-g-PEG rendered NCs with substantial stealth properties for both cell lines and for all the studied concentrations.

3.3. Biocompatibility.

To get a deeper insight on the interaction of the NCs with the two cell lines, the cell viability following incubation with increasing concentrations of PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs was evaluated. **Figure 7** shows a significant decrease in nCV only for RAW 264.7 cells in the presence of the highest concentration of NCs. In contrast, the results for HUVEC cells were very different and increasing nCV readings were observed for increasing amounts of NCs. We suggest that this

decrease in CV for RAW 264.7 could be a result of the NCs being engulfed by the cells instead of associated with the cell membrane. As opposed to HUVEC cells, macrophages are specialized cells that engulf and digest foreign substances. Enhanced CV as shown for HUVECs, has also been observed for other HBOCs following incubation with cells.³⁶ Such an enhanced CV was attributed to the O₂ carrying properties of the encapsulated Hb, thereby supplying O₂ to the cell environment and subsequently facilitating cell viability.

In addition to macrophages and endothelial cells, the interaction of nanoparticles with blood cells is an important factor to consider when fabricating NCs to be intravenously administered. The impact of both uncoated PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs and PEGylated PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs on RBCs was assessed by a hemolysis rate test. The Hb-loaded NCs showed haemolysis rates well below 5% for both NCs at all studied concentrations (**Figure 8A**). This is an important fact since biomaterials are considered non-hemolytic for haemolysis rates lower than 5%.¹¹ Photographic images of the collected supernatants shows a red coloration only for the positive control, indicating the presence of free Hb due to erythrocyte haemolysis (Figure 8B). DIC imaging furthermore shows no apparent change in the blood cells morphology following incubation with PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs as compared to the negative control (Figure 8C). Interestingly, even though no apparent effect on the haemolysis rate was observed following incubation of the uncoated PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs with the blood cells, NCs appeared as aggregates in the cell suspension and associated with the blood cells. The photographic insets show the suspensions of both NCs after overnight storage. While PEGylated PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs remain as a monodisperse suspension, sedimentation takes place for the uncoated PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs. These results highlight again the importance of the PEG coating which renders low-fouling properties (Figure 6) but also eliminates the NCs aggregation during storage and in presence of cells (Figure 8C).

3.4. Catalytic Activity.

After completing the assembly of the PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs, the catalytic activity as a result of the incorporation of CeO₂-NPs was evaluated. **Figure 9** shows the SOD-like and the combined SOD- and CAT-like activities for the Hb-loaded NCs after each deposition step. In a similar way as when evaluating the catalytic properties of the CeO₂-NPs in section 3.1.2, the SOD-

like activity of the NCs was assessed by the WST-1 assay. Figure 9A shows the nAbs readings at three different time-points. For PLGA-NPs, no decrease in nAbs was observed following incubation for 30 and 60 min. In contrast, upon PDA coating a ~25% decrease in nAbs is observed for the two longest time-intervals. This is not surprising since the antioxidant properties of PDA scavenging various radicals, ROS, and reactive nitrogen species have been previously demonstrated by us,^{27,36} and others.^{33,35} Next, deposition of PDA-coated Hb^{PDA} shows additional O₂^{•-} scavenging as shown by a 50% reduction in nAbs as compared to the controls, again highlighting the antioxidant properties of PDA. Importantly, deposition of the CeO₂-NPs onto the NCs resulted in almost complete depletion of O₂^{•-} as shown by a ~90% reduction in nAbs. Following PEGylation, no additional decrease in nAbs could be detected.

Following on, the CAT-like activity of the NCs was investigated by the Amplex Red assay (Figure 9B). With this assay, we first take advantage of the SOD-like activity of the NCs in catalyzing the decomposition of O₂^{•-} into H₂O₂. Next, we measure the scavenging potential of the NCs towards H₂O₂. No antioxidant activity was observed for the PLGA-NPs (Figure 9Bi). Interestingly, coating with PDA results on a slight increment in nFI, indicating increase in H₂O₂ concentration. Although such an increase is not significant, this trend has been previously reported in our group,²⁷ and could possibly be explained by oxidation of PDA in O₂-rich solutions, which produces H₂O₂ as an O₂-reduction product.⁵⁵ Once PDA is in the oxidized state, however, it loses the ROS-generating ability while still retaining radical-scavenging activities.⁵⁵ The Hb^{PDA} layer decreases the nFI continuously over time, with only 15% nFI left after 60 minutes. This decrease is thought to be due to the protective PDA coating on the Hb^{PDA}, which protects Hb against H₂O₂ (**Figure S4**), and has previously been employed to scavenge ROS in HBOCs systems.^{33,34} We speculate that the different behaviour of the PDA coatings is a result of the different underlying compounds (i.e., PLGA-NPs or Hb). We hypothesize that, as a result of the O₂ carrying abilities of Hb, the PDA coating could be in an oxidized state prior to incorporation into the NCs and, thus, displays only radical-scavenging properties. Importantly, similar to Figure 9A, the CeO₂-NPs are responsible for the depletion of the remaining ROS. The decrease in nFI after deposition of the CeO₂-NPs was furthermore compared to the activity of the free CeO₂-NPs. Performing the Amplex Red assay on both the free CeO₂-NPs and PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs showed 49% of the activity was retained after incorporating the CeO₂-NPs. While there is a decrease in activity, the CeO₂-NPs were still able to completely remove the ROS, as shown by a decrease in nFI to zero. Thus, these

results highlight the robust ability of CeO₂-based nanozymes to deplete ROS such as O₂^{•-} and H₂O₂.

Next, we evaluated whether the PEGylated PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs were able to conduct multiple rounds of catalytic activity. Since HBOCs are expected to circulate in the blood for an extended period of time, being able to deplete ROS in a continuous manner will prevent Hb oxidation into metHb. Figure 9Bii shows that on a first cycle, the PEGylated NCs were able to deplete 75% of H₂O₂ as shown by the decrease in nFI. On a second cycle, 66% of the total H₂O₂ had been depleted, showing a loss of 9% in catalytic activity. However, after the five subsequent cycles, the NCs were still able to preserve 42% of catalytic activity. This demonstrates that the developed Hb-loaded NCs are a robust system able to conduct multiple catalytic rounds, even after being subjected to several spinning and resuspension cycles. Together with the storage stability of the CeO₂-NPs (Figure 3B, C), this data shows the potential of the nanozymes in replacing the RBC's native antioxidant system in HBOCs. We envision that the prolonged H₂O₂ and O₂^{•-} scavenging will protect the encapsulated Hb against metHb conversion, thereby supporting the potential of the synthesized NCs as oxygen carriers.

4. CONCLUSIONS

A multi-layered nanocarrier was fabricated and evaluated in the context of oxygen delivering systems. The LbL technique was employed to adsorb different functional components (i.e., Hb^{PDA}, CeO₂-NPs, and PLL-g-PEG) onto a PLGA-NP core. Such combination created a nanocarrier able to reversibly bind and release O₂, deplete ROS, and show low-fouling properties.

It was demonstrated that Hb could be coated with PDA without altering the structure and functionality of the protein. CeO₂-NPs were synthesized and their ability to deplete O₂^{•-} and H₂O₂ was demonstrated prior and following incorporation into the nanocarrier. Cryo-TEM imaging was employed to confirm their presence in the nanocarrier system. The long storage stability combined with the catalytic activity over multiple cycles of ROS-scavenging show the CeO₂-NPs' potential as antioxidant system in the new generation of HBOCs. The surface modification using PLL-g-PEG resulted in lowered protein adsorption as well a significant reduction in cell association. Finally, the nanocarriers' biocompatibility was demonstrated by cell viability and hemocompatibility tests. Thus, in summary, the fabricated HBOCs can supply oxygen while

simultaneously providing a robust catalytic system, showing their potential as oxygen delivery systems.

FIGURES

Schematic 1. Schematic illustration of the LbL assembly of Hb-loaded NCs. PLGA-NPs were coated with PDA, PLL, Hb^{PDA}, PLL, CeO₂-NPs, and PLL-g-PEG. Hb is incorporated into the NCs to deliver O₂ to cells, while the CeO₂-NPs scavenge reactive oxygen species such as superoxide radical (O₂^{•-}) and hydrogen peroxide (H₂O₂), which are converted into O₂ and H₂O. PEGylation is employed to reduce or eliminate protein (e.g., serum albumin and IgG) adsorption.

Figure 1. Fabrication and characterization of Hb^{PDA} . A) Photographic images of the PDA deposition onto Hb over time as a result of the self-polymerization of DA. B) Hydrodynamic size distribution of bare Hb and Hb^{PDA} . C) FTIR spectra of Hb, Hb^{PDA} , and PDA only. D) CD spectra of Hb, Hb^{PDA} , and PDA only. As controls, partially denatured Hb (Hb at 60 °C) and fully denatured Hb (Hb at 100°C) were considered.

Figure 2. Oxygen carrying capacity of Hb^{PDA}. A) UV-Vis spectra of Hb^{PDA} following several cycles of purging with O₂, N₂, and CO. The table shows the band wavelengths of the references (i.e., oxygenated Hb (oxy-Hb), deoxygenated Hb (deoxy-Hb), and CO-bound Hb (CO-Hb)) and the Hb^{PDA} samples. B) ODCs of Hb and Hb^{PDA} with the corresponding p50 (the O₂ partial pressure (pO₂) at which 50% of Hb is saturated with O₂) and Hill coefficient (*n*) values.

Figure 3. Characterization of CeO₂-NPs. A) The hydrodynamic size, PDI, and ζ-potential. B) TEM image of the CeO₂-NPs. C) TEM phase contrast image of single CeO₂-NP showing crystallinity. D) TEM-SAD image with annotated diffraction rings.

Figure 4. Enzymatic activity of CeO₂-NPs. A) SOD-like activity of CeO₂-NPs over time measured by the WST-1 assay. i) O₂ is converted into O₂^{•-} by the xanthine/ XO system. O₂^{•-} oxidizes the WST-1 reagent into formazan which can be detected by UV-Vis at 450 nm. Due to the SOD-like activity of CeO₂-NPs, O₂^{•-} is consumed which results in a decrease of the amount of oxidized formazan. The activity of CeO₂-NPs versus native SOD (ii) and stability of the SOD-like activity of the CeO₂-NPs stored at 4 °C (iii). B) CAT-like activity of CeO₂-NPs over time measured by the Amplex Red assay. i) H₂O₂ interacts with the CeO₂-NPs to be converted, as a result of their CAT-like activity, into H₂O and O₂. H₂O₂ acts as a co-substrate for the conversion of the Amplex Red reagent into the fluorescent resorufin product in the presence of the enzyme HRP. The activity of CeO₂-NPs versus native CAT (ii) and stability of the CAT-like activity of the CeO₂-NPs stored at 4 °C (iii).

Figure 5. LbL assembly of the Hb-loaded NCs. A) The ζ -potential measurements following subsequent deposition of PDA, PLL, Hb^{PDA}, PLL, CeO₂-NPs, and PLL-g-PEG onto the PLGA-NPs. Inset shows color change of PLGA-NPs after PDA coating. B) Imaging of the final Hb-loaded NCs using DIC microscopy (i) and cryo-TEM (ii). The cryo-TEM shows the presence of individual CeO₂-NPs (black arrows) and a CeO₂-NPs aggregate (open arrows).

Figure 6. Evaluation of the stealth coating of the Hb-loaded NCs. A) FI readings of PEGylated PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs and uncoated PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs following incubation for 4 h at 37 °C with BSA^F and IgG^F. The FI of the NCs without protein exposure were measured as controls. B) CUE of RAW 264.7 (i) and HUVEC (ii) cells exposed to increased amounts NCs. C) nCMFI of RAW 264.7 (i) and HUVEC (ii) cells exposed to increased amounts of NCs. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$, PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs vs PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs.

Figure 7. Cell viability in presence of the Hb-loaded NCs. nCV readings of RAW 264.7 and HUVEC cells following incubation with increasing concentrations of PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs for 4 h. * $p \leq 0.05$, NCs vs Cells only.

Figure 8. Hemocompatibility of the Hb-loaded NCs. A) Hemolysis rate of uncoated PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs and PEGylated PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs at different concentrations. MQ and PBS were used as positive and negative controls, respectively. B) Photographic images of the supernatants of the two controls, the PLGA/Hb^{PDA}/(CeO₂-NPs)-NCs (i), and PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs (ii). C) DIC images of blood cells incubated with the NCs. Insets show the NCs suspensions after overnight storage at 4 °C.

Figure 9. Catalytic activity of Hb-loaded NCs. A) SOD-like activity measured as the conversion of O₂⁻ into H₂O₂ using the WST-1 assay. nAbs readings measured after each deposition step for three time intervals. PLGA-NPs are coated with PDA followed by the deposition of Hb^{PDA}, CeO₂-NPs, and PLL-g-PEG. B) Combined SOD-like and CAT-like activity evaluated by the Amplex Red assay. First, the NCs catalyze the decomposition of O₂⁻ into H₂O₂, followed by the subsequent decomposition of H₂O₂ into O₂ and H₂O. H₂O₂ acts as a co-substrate for the conversion of the Amplex Red reagent into the fluorescent resorufin product in the presence of the enzyme HRP. nFI readings due to the fluorescent resorufin product after each deposition step of the NCs assembly for three different time intervals (i). Recyclability of the combined SOD- and CAT-like activity of the complete PLGA/Hb^{PDA}/(CeO₂-NPs)/PEG-NCs for five subsequent cycles (ii).

ASSOCIATED CONTENT

Supporting Information.

Method section for the Hb extraction, synthesis of PLGA-NPs, BSA^F and PLL-g-PEG; Detailed characterization methods for Hb^{PDA}, CeO₂-NPs, and LbL assembly of the NCs; Quantification of CeO₂-NPs using UV-Vis and ICP-MS; saturation curves obtained by ζ -potential measurements for the deposition of the subsequent layers during the NC assembly; Oxygen binding reversibility of Hb^{PDA} over time; Oxidative protection of Hb^{PDA} against H₂O₂.

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