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Comprehensive biophysical and biochemical characterization of polymerized dopamine coated hemoglobin based oxygen carrier

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Abstract

Hemoglobin (Hb)-based oxygen carriers (HBOCs) are promising red blood cell (RBC) substitutes, when blood is unavailable. However, HBOCs can generate reactive oxygen species (ROS) species leading to oxidative stress, lipid peroxidation, DNA damage, and cell death. To address these issues, researchers have explored coating Hb and HBOCs with polymerized dopamine (PDA), to impart antioxidant properties via radical scavenging. This study builds upon previous PDA-coated Hb (PDA-hHb-1) research by quantifying HBOC biophysical properties and proposing two new PDA coating methods: 1) first binding carbon monoxide (CO) to human Hb (hHb) prior to PDA-coating to reduce metHb formation (PDA-hHb-13), and 2) forming PDA nanoparticles (NPs) before coating them with HbCO (PDA-hHb-15). After CO removal, PDA-hHb-13 and PDA-hHb-15 exhibited lower metHb levels (< 10 %) compared to PDA-hHb-1 (~13 %). PDA-hHb-15 had a reduced auto-oxidation rate constant compared to PDA-hHb-13, though both exceeded the precursor hHb. Functionally, PDA-coated hHbs retained similar oxygen offloading kinetics, with PDA-hHb-13 showing a lower oxygen affinity. PDA-coated hHbs exhibited enhanced ferric reducing and radical scavenging activity, but also increased ferryl species formation in the presence of H₂O₂, likely due to reduced catalase activity. Overall, PDA-coated Hb variants show promise as HBOCs with enhanced antioxidant properties, though further optimization is needed to balance stability and oxidative resilience.

Keywords

Hemoglobin; Hemoglobin-based oxygen carriers; Polydopamine-coated hemoglobin; Reactive oxygen species; Ferryl hemoglobin; Antioxidant activity

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Disclaimer

None

Declaration of competing interest

A.F.P. is an inventor on US patent application # PCT/US2023/079410 that describes the synthesis of PDA encapsulated Hb.

1. Introduction

Blood transfusions are critical for life-saving procedures, particularly during surgery, trauma management, and the treatment of hemolytic disorders [1,2]. However, the global demand for blood often exceeds the available supply due to multiple factors, including donor reluctance, cultural or religious restrictions, logistical challenges, and the sudden need arising from a mass casualty event [3]. Conventional transfusion practices also present low probability risks, such as exposure to blood-borne pathogens (e.g., viral hepatitis and HIV), blood type incompatibility, and complications from storage-induced alterations in red blood cells (RBCs), collectively termed the “storage lesion” [4]. To address these limitations, researchers have explored artificial oxygen carriers (AOCs), such as hemoglobin-based oxygen carriers (HBOCs) and perfluorocarbon-based oxygen carriers (PFCs) for use as RBC substitutes [5,6].

HBOCs, the most extensively studied AOC, gained significant interest in transfusion medicine during the 1980s and 1990s as potential RBC substitutes [7]. Early development efforts focused on utilizing stroma-free hemoglobin (Hb) due to its intrinsic oxygen-binding properties. However, cell-free Hb outside the protective environment of the RBC is inherently unstable, with the tetrameric Hb molecule rapidly dissociating into $\alpha\beta$ dimers, which can extravasate into the tissue space to elicit vasoconstriction, systemic hypertension, and iron-mediated oxidative tissue injury [8–10].

For HBOCs to achieve clinical efficacy, they must meet three essential criteria: efficient oxygen delivery to target tissues, prolonged intravascular retention, and minimal adverse effects, including vasoconstriction and oxidative toxicity. To address these challenges, researchers have employed various strategies to chemically or genetically modify Hb, improving its structural stability and oxygen affinity. These strategies include polymerization, polyethylene glycol surface conjugation, particle encapsulation, and genetic engineering, which aim to minimize Hb extravasation into tissue space and optimize its’ biophysical and functional properties [11].

Polymerization of Hb using glutaraldehyde has led to commercial HBOCs such as PolyHeme (Northfield Labs, Evanston, IL, USA) and Hemopure (Biopure Corp.) [12,13]. Cross-linking techniques have also been applied in the development of HBOCs, including HemAssist (Baxter, IL, USA) and Hemolink (Hemosol, Toronto, Canada) [14–16]. Surface conjugation methods, particularly through PEGylation, have resulted in commercial HBOCs such as Hemospan (Sangart, San Diego, CA, USA) and Sanguinate (Prolong, South Plainfield, NJ, USA) [17–20]. Additionally, genetically engineered Hb approaches have produced HBOCs such as Optro (Somatogen Inc.) [21,22]. However, none of these HBOCs achieved FDA approval due to safety concerns [23,24].

Preclinical and clinical investigations of various HBOCs have highlighted significant adverse effects, including nitric oxide (NO) scavenging, hyperoxygenation, and iron-associated toxicity [25]. Iron-mediated toxicity, in particular, remains a major concern due to its potential to induce oxidative stress, inflammation, and cellular damage [26]. The iron atom in Hb facilitates various redox reactions contributing to its reactivity. For instance,

the auto-oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) in Hb triggers a cascade of reactions that generate reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) [27]. H_2O_2 can further react with Fe^{2+} and Fe^{3+} through Fenton chemistry, generating unstable ferryl species (Fe^{4+}) which are highly reactive [27]. ROS are well-established mediators of lipid peroxidation and DNA degradation underscoring their central role in cellular injury and dysfunction [28,29].

Fortunately, RBCs possess a robust antioxidant system that includes superoxide dismutase (SOD), peroxiredoxin (PRDX), catalase (CAT) and glutathione peroxidase (GPx). This enzymatic system maintains the iron atom in Hb predominantly in its Fe^{2+} state, with less than 1 % of the total Hb within the RBC existing as methemoglobin (metHb) under normal physiological conditions [26]. Therefore, incorporation of antioxidants into the HBOC is a promising strategy for the development of oxidatively stable HBOCs. Various HBOC antioxidant strategies have been investigated in the literature. For example, Chang's group has synthesized PolyHb-SOD-CAT by α -polymerization of SOD and CAT with Hb [30]. Erythromer developed by Kalocyte encapsulates leucomethylene blue along with Hb to reduce metHb formation [31]. Additionally, Hosta-Rigau's group has investigated metal phenolic network (MPN) coatings for HBOCs to impart antioxidant protection [32]. Several groups have explored polydopamine (PDA) coating of Hb to impart antioxidant properties to the HBOC. PDA is synthesized through the self-polymerization of dopamine, a neurotransmitter. It is well known for its biocompatibility, biodegradability, and versatility in adhering to a wide range of surfaces [33]. PDA reacts with ROS and breaks it down into smaller less toxic degradation products, serving as an antioxidant layer to protect against protein and lipid oxidation [34]. PDA degradation products can also be internalized by cells, where they can further quench intracellular ROS [35].

Wang et al. 2017 coated bovine Hb (bHb) with PDA through dopamine polymerization at basic pH [36], and demonstrated that bHb retained its' structural and functional properties post-coating. The coated bHb exhibited enhanced antioxidant activity effectively quenching free radicals as well as intracellular ROS, while maintaining hemocompatibility. Similarly, Hu et al. 2017 synthesized PDA-Hb microparticles and reported enhanced oxygen release capability, oxidative protection, and hemocompatibility of the microparticles [37]. More recently, Chen et al. 2021 performed PDA-coating of Hb nanoparticles, and Pozy et al. 2023 performed PDA-coating of *Lumbricus terrestris* erythrocrucorin (LtEc), further validating these findings [38,39]. Collectively, these studies highlight the enhanced antioxidant activity conferred by PDA coatings, including the scavenging free radicals and ROS; as well as, the conversion of metHb to functional ferrous Hb due to PDA's redox properties. However, further research is needed to assess the stability of the Hb post coating, specifically regarding metHb formation, auto-oxidation, and higher redox state (ferryl Hb) kinetics.

In this study, we synthesized PDA-coated human Hb (hHb) by the traditional method at basic pH (referred to as PDA-hHb-1). To mitigate the formation of metHb, we pre-bound carbon monoxide (CO) to hHb (hHbCO) before coating it with PDA, followed by purification and CO-unlocking prior to analysis (referred to as PDA-hHb-13). Additionally, we developed a two-step reaction method in which dopamine was first polymerized into PDA nanoparticles, and subsequently coated with HbCO, producing PDA-hHb-15. The

PDA-hHb variants were assessed by quantifying metHb content, auto-oxidation kinetics, and ferryl Hb kinetics. The influence of the PDA coating on the secondary and tertiary structure of hHb, as well as the stability of the protein, was evaluated using circular dichroism (CD) spectroscopy. Equilibrium oxygen-binding parameters, including oxygen affinity (P_{50}) and cooperativity (n_{50}), were assessed using a Hemox Analyzer, while oxygen dissociation and haptoglobin binding kinetics were measured via stopped-flow spectroscopy. Antioxidant properties were assessed by the ferric reducing antioxidant power (FRAP) activity assay, (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (ABTS) radical scavenging assay, sulfhemoglobin (sulfHb) formation assay, and catalase activity assay.

2. Material and methods

2.1. Materials

Sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), dopamine HCl ($\text{C}_8\text{H}_{12}\text{ClNO}_2$), iron chloride (FeCl_3), ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were acquired from Sigma-Aldrich (St. Louis, MO). Sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium hydroxide (NaOH), potassium cyanide (KCN), and potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) were acquired from Fisher Scientific (Pittsburgh, PA). 2,4,6-tripyridyl-*S*-triazine (TPTZ) was obtained from TCI Chemicals (Portland, OR). Recently expired human RBC units were acquired from Impact Life (Davenport, IA). Polysulfone (PS) hollow fiber filter modules with a pore size of 50 kDa (D02-S050-05-N) and modified polyether sulfone (mPES) modules with pore sizes of 50 kDa (N04-S050-05-N), 500 kDa (N04-S500-05-N), and 0.65 μm (N04-E65U-07-N) were acquired from Repligen Corp. (Rancho Dominguez, CA). Gas-liquid membrane contactors (3M™ Liqui-Cel™ MM 1 $\times$ 5.5 Series Membrane Contactor G543) were purchased from Solvntum (St. Paul, MN). Ultraviolet (UV) lamps (UVP Blak-Ray B-100AP) were acquired from Analytik Jena (Upland, CA).

2.2. Human Hb purification and quantification

Human Hb (hHb) was purified from expired RBC units as reported by Khan et al. 2024 [40]. Briefly, expired packed human RBC units were washed with 0.9 wt% saline solution via tangential flow filtration (TFF) over a 0.65 μm mPES hollow fiber cartridge for 6 diafiltration cycles. Washed RBCs were subsequently lysed with a hypotonic phosphate buffer (PB) (3.75 mM, pH 7.4). Human Hb was extracted from the RBC lysate as the permeate of a 500 kDa hollow fiber cartridge, using TFF. The purified hHb was further concentrated using a 50 kDa hollow fiber cartridge and stored at -80°C until further use. A diode array UV-visible spectrophotometer HP 8452 A (Olis, Athens, GA) was used to quantify the concentration of purified hHb using the cyanomethemoglobin assay, as described in Section 2.4 [41].

2.3. Synthesis and purification of PDA-hHb

Three distinct groups of PDA-coated hHb were synthesized using different methodologies, with each synthesis performed in triplicate. The synthesis of PDA-hHb-1 followed methods described in the literature [33,36]. Briefly, 97.6 mg dopamine-HCl and 152 mg hHb were simultaneously added to 20 mL 10 mM Tris buffer (pH 8.5) at room temperature. The

reaction proceeded for 1 h, after which the resulting PDA-hHb-1 was purified through repeated washing (4 times) using a 50 kDa MWCO Amicon[®] ultra centrifugal filter (Millipore Sigma) to remove unreacted dopamine and to perform buffer exchange to a physiological pH, using 155 mM phosphate-buffered saline (PBS, pH = 7.4).

PDA-hHb-13 was synthesized using a one-step synthesis method, which involved pre-binding hHb with carbon monoxide (CO) via a gas-liquid contactor (3M[™] Liqui-Cel[™] MM 1 × 5.5 Series Membrane Contactor G543), followed by PDA coating under similar conditions to those used for synthesizing PDA-hHb-1. The reaction was scaled up to yield sufficient protein for comprehensive characterization. Briefly, 642 mg of dopamine-HCl and 1000 mg hHbCO were simultaneously added to 132 mL 10 mM Tris buffer (pH 8.5) at room temperature. After 1 h of reaction, the resulting PDA-hHb-13 (CO) was purified via TFF with a 50 kDa cartridge over 8 diafiltration cycles with 155 mM PBS (pH = 7.4) and stored at −80 °C until further analysis. The bound CO in PDA-hHb-13 was subsequently removed (CO-unlocked) via photolysis, and the metHb content of the non-CO-bound PDA-hHb-13 was determined, as described in Section 2.4.

The synthesis of PDA-hHb-15 utilized a two-steps synthesis method, where dopamine was first polymerized into PDA nanoparticles before coating it with hHbCO as shown in Scheme 1. Initially, 642 mg dopamine-HCl was added to 132 mL of 10 mM Tris buffer (pH 8.5) at room temperature. After 30 mins of polymerization, 1000 mg hHbCO was introduced, and the reaction was continued for an additional 30 min. Following a total reaction time of 1 h, the resulting PDA-hHb-15 (CO) was purified via TFF using a 50 kDa cartridge over 8 diafiltration cycles with 155 mM PBS (pH = 7.4) and stored at −80 °C until further analysis. CO-unlocking and metHb assay were performed prior to biophysical characterization.

2.4. CO-unlocking and MetHb assay

CO-unlocking of PDA-hHb was performed to generate oxygenated PDA-hHb for subsequent analysis. A solution (10 mL) of PDA-hHb-13 (CO-locked) or PDA-hHb-15 (CO-locked) at a hHb concentration of 7 mg/mL was placed in a rotating round bottom flask and irradiated using UV lamps (UVP Blak-Ray B-100AP) in an oxygen-rich environment, at room temperature for 5 min to photolyze any bound CO (Scheme 2). The efficiency of CO removal was evaluated via spectral deconvolution, by quantifying the percentage of oxyHb, deoxyHb and HbCO in the photolyzed sample.

MetHb content and the total Hb concentration of hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 were quantified using a modified cyanomethemoglobin assay [41]. Briefly, protein samples were diluted to about 10 mg/mL and the absorbance of the samples was measured before and after the addition of 10 μ L of 10 % (w/v) potassium cyanide (KCN). The decrease in absorbance at 630 nm upon addition of KCN was used to quantify metHb content. The absorbance at 700 nm was used for baseline correction, to ensure the absorbance difference at 630 nm was solely due to metHb and not from light scattering effects. To quantify the total Hb concentration, protein samples were diluted to about 1 mg/mL, followed by the addition of 10 μ L of 10 % (w/v) KCN and 10 μ L of 20 % (w/v) potassium ferricyanide ($K_3Fe(CN)_6$), which converts all Hb to cyanometHb. Total protein was quantified by quantifying cyanometHb, by measuring its absorbance at 540 nm.

2.5. Quantifying dopamine in the PDA coating

The amount of dopamine incorporated into the PDA coating was calculated based on a mass balance on dopamine, considering the initial mass of dopamine, the mass of dopamine removed during TFF purification of the PDA-Hb variant, and the mass of dopamine retained in the coating. A dopamine calibration curve was generated to calculate the extinction coefficient of dopamine at 280 nm in PBS. Dopamine removed during TFF purification was quantified by measuring the absorbance of the permeate at 280 nm and the volume of the permeate. The absorbance at 418 nm (Soret peak for HbCO) was used to estimate the contribution of hHb to the absorbance at 280 nm using the extinction coefficient of hHb at the two wavelengths. The corresponding dopamine mass was determined using the dopamine extinction coefficient and the total volume of the permeate after each TFF diacycle (Eq. (1)). The mass of dopamine incorporated in the coating was calculated using Eq. (1).

$$\text{Hb Absorbance at 280 nm} = (\text{Hb Absorbance at 418 nm}) * \frac{\text{Extinction Coefficient of HbCO at 280 nm}}{\text{Extinction Coefficient of HbCO at 418 nm}}$$

Dopamine absorbance at 280 nm = Total absorbance at 280 nm – Hb absorbance at 280 nm.

For TFF diacycle i ,

$$D_i = \frac{\text{Dopamine Absorbance at 280 nm} * \text{Dilution Factor} * \text{Permeate Volume}}{\text{Dopamine Extinction Coefficient at 280 nm}}$$

Dopamine incorporated in the PDA coating was calculated as:

$$\text{Dopamine in PDA coating} = D_a - \sum D_i \quad (1)$$

where, D_a = initial mass of dopamine, D_i = mass of dopamine permeated in TFF diacycle i .

2.6. Dynamic light scattering (DLS)

The hydrodynamic diameter of the hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 was measured using dynamic light scattering (DLS). Protein samples were diluted to ~0.5 mg/mL in deionized water, and the measurement were performed with a Zetasizer Pro (Malvern Panalytical Inc., Westborough, MA) at a wavelength of 637 nm and a scattering angle 90°. The reported hydrodynamic diameter represents the volume-weighted average of three measurements.

2.7. Oxygen equilibrium curves

Oxygen equilibrium curves (OECs) for the proteins were measured using a Hemox Analyzer (TCS Scientific Corp. New Hope, PA) at 37 °C and pH 7.40. Proteins were diluted to a concentration of 60 µM (heme basis) in Hemox buffer supplemented with 20 µL additive A and 20 µL antifoam agent (TCS Scientific Corp.). The resulting data were fit to the Hill

equation to regress the partial pressure of O₂ at which 50 % of Hb binding sites are saturated with O₂ (P₅₀) and the Hill coefficient (n₅₀) a measure of cooperative oxygen binding.

2.8. Auto-oxidation kinetics

The spontaneous conversion of hHb to metHb was measured using UV–visible spectroscopy as described in the literature [42]. Briefly, oxygenated protein samples were diluted to 12.5 mg/mL (Hb basis) and incubated at 37 °C and pH 7.4 for 20 h. Spectra were collected at 30 min intervals and metHb content was quantified from the absorbance at 630 nm. The auto-oxidation rate constant ($k_{\text{auto-ox}}$) was calculated by fitting the kinetics of ferrous Hb concentration to an exponential function.

2.9. Stopped-flow spectroscopy

Kinetic measurements of oxygen offloading and haptoglobin binding were performed using a SX-20 microvolume stopped-flow instrument (Applied Photophysics, Charlotte, NC) as described in the literature [42].

2.9.1. Oxygen offloading kinetics: Oxygenated protein samples (hHb, PDA-hHb-1, PDA-hHb-13, PDA-hHb-15) were diluted to 12.5 μM (Hb basis) and mixed with 1.5 mg/mL of sodium dithionite in PBS to initiate oxygen offloading. The kinetics of oxygen offloading were monitored at an absorbance of 437.5 nm for 60 s for each time course. The oxygen dissociation rate constant ($k_{\text{O}_2,\text{off}}$) was derived from fitting at least 7 kinetic traces to a first-order rate law.

2.9.2. Haptoglobin binding kinetics: Haptoglobin (Hp) at 0.25 μM was mixed with varying Hb concentrations (5, 10, 15, and 20 μM : Hb basis). Hp binding to heme protein was monitored via fluorescence quenching of the Hp at an emission wavelength of 310 nm by the heme groups in the Hb protein. For each protein concentration, at least 7 kinetic traces were fit to single exponential function to obtain the pseudo-first order binding rate constant (k_{obs}) in units of s^{-1} . The bimolecular binding rate constant ($k_{\text{Hp-Hb}}$) in units of $\mu\text{M}^{-1} \text{s}^{-1}$ was derived from the slope of k_{obs} versus protein concentration.

2.10. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay quantifies the reducing power by measuring the reduction of Fe^{3+} to Fe^{2+} and was performed following the protocol described in the literature [43]. Briefly, a working solution containing acetate buffer (300 mM, pH = 3.6), TPTZ solution (10 mM in 40 mM HCl), and FeCl_3 (20 mM) in a 10:1:1 volume ratio was prepared. A standard curve was generated using ascorbic acid (AA) as a reference compound. To establish the curve, 10 μL of varying concentrations of AA (0, 50, 100, 250, 500, 1000 μM) were added to 200 μL of the working solution in a 96-well microplate. The mixture was incubated at 37 °C for 4 min, and the absorbance was measured at 593 nm. The absorbance values were plotted against AA concentration to generate the standard curve. Protein samples at concentrations of 2 mg/mL (Hb basis) were analyzed under the same conditions. The FRAP value for each sample was calculated using Eq. (2), based on the relationship defined by the standard curve.

$$\text{Protein FRAP value}(\mu\text{M}/\mu\text{g protein}) = \frac{2 * [\text{Abs}(\text{WS} + \text{sample}) - \text{Abs}(\text{WS} + \text{PBS}) - \text{Abs}(\text{PBS} + \text{sample})]}{10(\mu\text{L}) * \text{sample concentration} * \text{calibration curve slope}} \quad (2)$$

where, WS = working solution, PBS = phosphate-buffered saline.

2.11. ABTS radical scavenging assay

The ABTS assay assesses the antioxidant activity of a compound to reduce free radicals by quantifying the power to reduce the ABTS radical (ABTS[•]). The method for the assay were adapted from methods in the literature [44]. A working solution was prepared by adding 7 mM ABTS to sodium periodate (NaIO₄, 4.9 mM) to generate oxidized ABTS[•] radicals, and stored in the dark for at least 14 h before use. A working solution was prepared by diluting the ABTS stock solution to yield 0.1–1.0 mAu absorbance at 734 nm. 10 μL of protein samples (diluted to 2 mg/mL) were mixed with 200 μL of the working solution. The ABTS reference (ABTS Ref) was prepared similarly by mixing 200 μL of working solution with 10 μL PBS. All samples were incubated in the dark for 5 mins at room temperature, and the free radical scavenging ratios of the proteins were determined by monitoring the absorbance at 734 nm and using Eq. (3).

$$\text{Scavenging ratio}(\% / \mu\text{g protein}) = \frac{\text{Abs}(\text{ABTS Ref}) - (\text{Abs}(\text{ABTS} + \text{sample}) - \text{Abs}(\text{PBS} + \text{sample}))}{10(\mu\text{L}) * \text{sample concentration} * \text{Abs}(\text{ABTS Ref})} * 100 \quad (3)$$

2.12. Circular dichroism spectroscopy

The circular dichroism (CD) spectra for hHb and PDA-hHb variants were acquired using a JASCO J-815 CD spectrometer (JASCO, Easton, MD). To analyze secondary structure, far-UV CD spectra were recorded between 190 and 260 nm using protein samples at a concentration of 0.5 mg/mL in 3.7 mM PBS buffer (pH 7.4). A quartz cell with a 0.1 cm path length was used for these measurements. Near-UV CD spectra were recorded from 250 to 500 nm for tertiary structure analysis, using protein samples at 1.5 mg/mL in the same buffer and quartz cell. Each spectrum was averaged over three scans, with baseline corrections performed using 3.7 mM PBS buffer (pH 7.4). CD data were expressed as molar ellipticity (deg cm² dmol⁻¹). Differences in α-helical content due to the PDA coating were evaluated by comparing the α-helix peak at 222 nm across hHb and PDA-hHb variants.

2.13. Thermal stability

To evaluate the thermal stability, hHb and PDA-hHb variants were prepared at a concentration of approximately 0.5 mg/mL in 3.7 mM phosphate buffer (pH 7.4). Far-UV CD spectroscopy was used to monitor the ellipticity at 222 nm as the temperature was gradually increased. A quartz cell with a 0.1 cm path length was utilized for these measurements. The temperature was raised from 20 °C to 90 °C at a rate of 2.5 °C per minute, with a 3-min equilibration at each temperature step, controlled via a Peltier device

attached to the JASCO J-815 CD spectrometer. Baseline corrections were made using 155 mM PBS buffer (pH 7.4).

2.14. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were prepared at a concentration of 0.5 mg/mL and combined in a 1:1 volume ratio with tris-glycine SDS sample buffer (Novex, Carlsbad, CA). For reducing conditions, 0.1 M dithiothreitol (DTT) was included in the mixture. The samples were heated at 85 °C for 10–15 min using a heat block. A volume of 20 µL from each sample was loaded onto a 4–20 % tris-glycine gradient gel (Invitrogen, Waltham, MA) and subjected to electrophoresis in a Mini Gel Tank system (Invitrogen) for 40 min at 220–225 V. Following electrophoresis, the gels were stained with Coomassie blue for 20–45 min and subsequently destained using a solution comprising 60 % deionized water, 30 % methanol, and 10 % acetic acid for 1–2 h. Gel images were obtained, and molecular weight analysis was performed using a standard molecular weight ladder as a reference.

2.15. Hydrogen peroxide mediated oxidation

Oxidation of ferrous Hb (60 µM heme basis) with H₂O₂ (600 µM) was studied in a photodiode array spectrophotometer following the procedure described in the literature [45]. Precursor Hb and PDA-coated Hbs were incubated with H₂O₂ for 5 min and quenched with catalase (200 units/mL) for 1 min. 2 mM sodium sulfide (Na₂S) was added to convert ferryl Hb to sulfated Hb (sulfHb), which was quantified by its' absorbance at 620 nm (extinction coefficient 24 mM⁻¹ cm⁻¹).

2.16. Catalase activity assay

Catalase activity of the precursor hHb and PDA-coated hHbs was measured by methods reported in the literature [45]. A standard curve was generated by incubating catalase standards of varying concentrations (0, 1, 2, 3, 4, 5, and 10 units/mL) with 15 mM H₂O₂, and the absorbance was measured at 240 nm over 3 min. hHb and PDA-coated hHbs were analyzed similarly by incubating samples (2 µM Hb basis) with 15 mM H₂O₂. A comparison to the standard was performed to determine the catalase activity of the samples.

2.17. Statistical analysis

Data where applicable are reported as the average ± standard deviation. Statistical analysis was performed using JMP software (JMP Pro, Version 16. SAS Institute Inc., Cary, NC). The assumptions of the data following a normal distribution and having equal variances were validated with the Shapiro-Wilk test and the unequal variances test respectively. Tukey's Test was used to compare means following the analysis of variance (ANOVA) test with a significance level of 5 % ($\alpha = 0.05$).

3. Results

3.1. hHb purification and quantification of protein and methemoglobin level

Purification of the precursor hHb was performed using an established protocol in the literature [40]. Protein concentrations and methemoglobin (metHb) levels for the precursor

hHb and PDA-coated hHb (PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15) were quantified using the cyanomethemoglobin assay (Table 1). The concentration of the precursor hHb was determined to be 210 ± 0.8 mg/mL with a metHb level of 1.0 ± 0.1 %, while the protein concentrations for PDA-coated hHb samples were 7.5 ± 0.4 mg/mL for PDA-hHb-1, 24.3 ± 3.8 mg/mL for PDA-hHb-13 and 23.9 ± 1.4 mg/mL for PDA-hHb-15.

PDA-hHb-1 (literature method) demonstrated elevated metHb formation, with a metHb level of 13.7 ± 1.5 %. While previous studies involving PDA-Hb did not report precise quantification of metHb, the study by Nadimifar et al., 2023 observed a significant shift in the Soret peak of PDA-Hb to 405 nm, a spectral signature of metHb [46]. To address this, we synthesized PDA-hHb-13 and PDA-hHb-15, with the precursor hHb initially bound to CO prior to PDA coating to reduce metHb formation. The CO was subsequently removed by irradiating the PDA-hHb-13 and PDA-hHb-15 samples with UV light in an oxygen-rich environment, facilitating the displacement of CO and binding of oxygen.

MetHb levels were assessed both before and after the CO unlocking procedure. For the precursor hHb, the metHb level was the lowest at 1.0 ± 0.1 % ($p < 0.005$) compared to all PDA-hHb variants. PDA-hHb-1 showed the highest metHb level of all PDA-hHb variants, at 13.7 ± 1.5 % after purification ($p < 0.001$). PDA-hHb-13 (PDA coating of hHbCO) exhibited an initial metHb level of 2.7 ± 0.5 %, which increased to 4.4 ± 0.5 % following the CO unlocking procedure. PDA-hHb-15 (hHbCO coated with PDA NPs) showed a metHb level of 4.4 ± 0.3 % before unlocking, rising to 5.6 ± 0.4 % post-unlocking, which was not significantly different than the metHb level for PDA-hHb-13 post CO unlocking ($p > 0.05$).

Spectral deconvolution of CO-unlocked PDA-hHb-13 and PDA-hHb-15 samples revealed that more than 90 % of the hHb was present in the oxyHb form. To minimize spectral interference in downstream spectroscopic assays, a threshold of 5–10 % HbCO was established as acceptable ensuring that the remaining CO-bound fraction does not significantly affect spectral analyses. These findings suggest that the CO-unlocking procedure induced a minor but notable increase in metHb levels in both PDA-hHb-13 and PDA-hHb-15.

3.2. PDA-hHb purification

To assess the efficiency of the PDA-hHb purification process, the mass of dopamine permeated through the TFF membrane into the waste container after each diacycle of PDA-hHb-15 purification was calculated by measuring the permeate volume and its' associated UV–visible spectra. Fig. 1A shows the absorption spectra for dopamine, indicating an absorption maximum at 280 nm. A calibration curve for dopamine, shown in Fig. 1B, was generated by measuring the absorbance at 280 nm, which provided an extinction coefficient of 14.7 (mg/mL) $^{-1}$ cm $^{-1}$ for dopamine. This extinction coefficient was utilized to convert the absorbance value to dopamine concentration (mg/mL). The dopamine concentration was then multiplied by the permeate volume to calculate the mass of dopamine permeated during each TFF diacycle (Eq. (1)). An example permeate spectra is shown in Fig. 1C. It is important to note that there was negligible absorbance at 418 nm, indicating that hHb did not significantly permeate through the TFF membrane and therefore did not contribute to the 280 nm absorbance. Nevertheless, the absorbance at 418 nm was used to calculate the

contribution of hHb to the 280 nm absorbance by using the ratio of extinction coefficient of hHb at 280 nm to that at 418 nm. Since the hHb used was approximately 98 % pure [40], the contribution from other proteins to the 280 nm absorbance was considered negligible. The mass of dopamine permeated during the purification of PDA-hHb-15 is shown in Fig. 1D, which indicates that purification is completed by the fifth diacycle.

3.3. Quantification of dopamine in PDA coating

The dopamine content in the PDA coating of PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 was calculated via mass balance using the initial mass of dopamine added to the reaction, the dopamine permeated during TFF purification, and the dopamine present in the coating, and was reported as mg of dopamine per g hHb. The analysis revealed that PDA-hHb-1 contained 42.7 ± 19.6 mg dopamine per g of hHb. PDA-hHb-13 contained 34.3 ± 7.1 mg dopamine / g hHb, while PDA-hHb-15 contained 61.3 ± 27.7 mg dopamine / g hHb within their respective coatings. There was no statistical significance observed for the mass of dopamine in the coatings between the three groups ($p > 0.05$). The higher standard deviation observed for PDA-hHb-15, can be attributed to the synthesis process, during which dopamine was allowed to form PDA nanoparticles (PDA NPs). These PDA-NPs can scatter light and will interfere with absorbance measurements of the permeate. For instance, an absorbance contribution as small as 0.01 mAu can translate into a difference of 14 mg of incorporated dopamine when accounting for the total permeate volume of 500 mL and the applied dilution factors.

3.4. Dynamic light scattering (DLS)

The hydrodynamic diameter of the precursor hHb and PDA-coated hHbs (PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15) was measured using DLS, as shown in Fig. 2A and Table 1. The measured diameters were 3.7 ± 0.3 nm for hHb, 6.5 ± 1.3 for PDA-hHb-1 (literature method), 6.0 ± 1.0 nm for PDA-hHb-13 (PDA coating of hHbCO), and 385 ± 54 nm for PDA-hHb-15 (hHbCO coated with PDA NPs). PDA-hHb-15 exhibited a significantly larger diameter compared to hHb, PDA-hHb-1 and PDA-hHb-13 ($p < 0.001$), whereas the rest of the groups were not statistically different compared to each other ($p > 0.05$). This increase in size for PDA-hHb-15 stems from the PDA NPs formed in the first 30 mins of PDA-hHb-15 synthesis that were measured to be 110 ± 23 nm in size, according to DLS. The polydispersity index (PDI) for PDA-hHb-1 (0.07 ± 0.02) and PDA-hHb-13 (0.02 ± 0.0) was not significantly different than hHb (0.05 ± 0.02) ($p > 0.05$), while PDA-hHb-15 showed a significantly higher PDI at 1.25 ± 0.05 ($p < 0.001$). These results suggest that the two-step coating method used for PDA-hHb-15 results in particles with considerably larger dimensions and a higher polydispersity compared to hHb and the other PDA-Hb variants.

3.5. SDS-PAGE

The precursor hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 were analyzed by SDS-PAGE under both non-denaturing and denaturing conditions, as shown in Fig. 2B and C. Lane 11 displays the molecular weight (MW) marker, while lane 10 contains the precursor hHb. Lanes 9–7, 6–4 and 1–3 correspond to three batches of PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15, respectively. All samples, including the precursor hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15, exhibited a prominent band at approximately 16 kDa,

consistent with the expected MW of the α - and β -subunits of hHb. Under both gel conditions, the tetrameric hHb ($\alpha_2\beta_2$) dissociates into α/β monomeric subunits, which co-migrate to produce a single, intense band. Additionally, faint bands at approximately 30–32 kDa were observed, likely representing residual RBC enzymes, since the hHb precursor is 98 % pure. Notably, PDA-hHb-15 and PDA-hHb-1 displayed an additional higher molecular weight band with greater intensity under non-denaturing conditions, suggesting a higher degree of crosslinking with PDA compared to PDA-hHb-13. This higher MW band was still detectable, though with reduced intensity, under denaturing conditions, further supporting the higher extent of cross-linking in PDA-hHb-15 and PDA-hHb-1 compared to PDA-hHb-13.

3.6. Oxygen equilibrium curve

The oxygen equilibrium curves (OECs) of the precursor hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 were measured using a Hemox Analyzer to study the effect of the PDA coatings on the oxygen affinity of hHb (Fig. 3A). The P_{50} value, defined as the partial pressure of oxygen at which 50 % of Hb binding sites are saturated with oxygen, was determined to be 10.6 ± 0.6 mmHg for PDA-hHb-1, 17.2 ± 0.9 mmHg for PDA-hHb-13 and 13.4 ± 0.6 mmHg for PDA-hHb-15, compared to 14.5 ± 0.7 mmHg for unmodified hHb. These results indicate that PDA coating of hHb via the basic pH synthesis method reported in the literature (PDA-hHb-1) significantly decreased the P_{50} ($p < 0.001$) compared to hHb, while the one-step synthesis method with CO-locked hHb (PDA-hHb-13) significantly increased the P_{50} value ($p < 0.001$) compared to hHb. Conversely, the two-step method of PDA coating using pre-formed PDA NPs (PDA-hHb-15) did not result in a statistically significant alteration of the P_{50} compared to hHb ($p > 0.005$). The Hill coefficient, a measure of cooperative oxygen binding, was reduced to 1.3 ± 0.1 for PDA-hHb-1 ($p < 0.001$), 1.6 ± 0 for PDA-hHb-13 ($p < 0.001$) and 1.3 ± 0.1 for PDA-hHb-15 ($p < 0.001$), compared to 2.8 ± 0.2 for unmodified hHb. This indicates a significant decrease in the cooperativity of oxygen binding for hHb upon PDA coating, irrespective of the synthesis method employed.

3.7. Oxygen offloading kinetics

Oxygen offloading kinetics ($k_{O_2, \text{off}}$) for the precursor hHb and PDA-coated hHb variants were determined using stopped-flow UV–visible spectroscopy, and the kinetic traces are shown in Fig. 3B. Unmodified hHb exhibited a $k_{O_2, \text{off}}$ of $41.1 \pm 0.8 \text{ s}^{-1}$, which was comparable to the value observed for PDA-hHb-13 ($41.3 \pm 1.3 \text{ s}^{-1}$) with no statistical difference ($p > 0.05$). PDA-hHb-1 and PDA-hHb-15 showed reduced oxygen offloading rates of $30.2 \pm 3.2 \text{ s}^{-1}$ ($p < 0.01$) and $32.9 \pm 3.4 \text{ s}^{-1}$ ($p < 0.05$) respectively, compared to the precursor hHb.

3.8. Auto-oxidation kinetics

Under oxygenated conditions, ferrous (Fe^{2+}) Hb undergoes auto-oxidation to form ferric (Fe^{3+}) Hb. Ferric Hb is more prone to the formation of ferryl (Fe^{4+}) species, which leads to unfolding of the globin chains and release of heme [27]. Measuring the auto-oxidation rate constant of the precursor hHb and PDA-coated hHb variants is therefore crucial for assessing their oxidative stability. The auto-oxidation rate constants for hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 were determined as previously described in the literature

[42]. Representative kinetic traces are provided in Fig. 4, and the auto-oxidation rate constants ($k_{\text{auto-ox}}$) are summarized in Table 1. The auto-oxidation rate constants ($k_{\text{auto-ox}}$) for the precursor hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 were $0.008 \pm 0.001 \text{ h}^{-1}$, $0.075 \pm 0.009 \text{ h}^{-1}$, $0.079 \pm 0.006 \text{ h}^{-1}$, and $0.041 \pm 0.006 \text{ h}^{-1}$ respectively. The three PDA-coated variants exhibited significantly higher rates of auto-oxidation compared to the precursor hHb ($p < 0.001$). However, PDA-hHb-15 (coating by pre-formed PDA NPs) demonstrated a notably lower oxidation rate ($p < 0.001$), approximately half that of PDA-hHb-1 and PDA-hHb-13, indicating reduced generation of metHb. PDA-hHb-1 and PDA-hHb-13 had similar rate of auto-oxidation, with no statistical difference ($p > 0.05$). These results suggest that while the PDA coatings increase the auto-oxidation rate constant, PDA-hHb-15 may offer better performance with reduced formation of non-functional metHb compared to PDA-hHb-1 and PDA-hHb-13.

3.9. Haptoglobin binding kinetics

Hp is a plasma protein that plays a critical role in the clearance of cell-free Hb from the circulation [47]. To investigate the binding kinetics between Hp and hHb or PDA-hHb variants, a fixed concentration of Hp was titrated with varying concentrations of precursor hHb or PDA-hHb variants. The binding interaction was monitored via fluorescence quenching in a stopped-flow apparatus. The kinetic time course depicting changes in fluorescence upon binding of Hp to precursor hHb or PDA-coated hHb variants is shown in Fig. 5A. The bimolecular rate constant ($k_{\text{Hb-Hp}}$) was determined by plotting the pseudo-first-order rate constant as a function of hHb concentration (Fig. 5B and Table 1). The precursor hHb had a $k_{\text{Hb-Hp}}$ of $0.15 \pm 0.02 \mu\text{M}^{-1} \text{ s}^{-1}$. Comparatively, PDA-hHb-1 had a $k_{\text{Hb-Hp}}$ of 0.20 ± 0.03 , PDA-hHb-13 had a of $0.112 \pm 0.004 \mu\text{M}^{-1} \text{ s}^{-1}$, and that for PDA-hHb-15 was $0.124 \pm 0.012 \mu\text{M}^{-1} \text{ s}^{-1}$, with no statistical difference compared to the precursor hHb and with each other ($p > 0.05$). These results indicate that PDA coating of hHb does not significantly alter the Hp binding rate constant.

3.10. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was utilized to assess the antioxidant properties of the precursor hHb, PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15 (Fig. 6A and Table 1). This assay evaluates a protein's ability to convert ferric ions (Fe^{3+}) into ferrous ions (Fe^{2+}), as indicated by an increase in the absorbance at 593 nm, which corresponds to a higher Fe^{2+} concentration. The FRAP values per μg of protein, for the precursor hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 were $14.8 \pm 0.5 \mu\text{M}$, $34.7 \pm 2.7 \mu\text{M}$, $31.5 \pm 1.1 \mu\text{M}$, and $28.6 \pm 1.4 \mu\text{M}$, respectively. These results demonstrate that PDA-coated hHbs (PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15) had significantly greater antioxidant activity compared to the precursor hHb ($p < 0.001$). PDA NPs (without hHb) showed a FRAP value of $63.2 \pm 6.1 \mu\text{M}$, confirming that the increased FRAP value for the PDA-hHbs was due to PDA. However, there was no significant difference in FRAP activity amongst the PDA-coated hHbs ($p > 0.05$). This indicates that the PDA coating effectively enhances the reducing power of hHb, improving its ability to convert Fe^{3+} to Fe^{2+} .

3.11. ABTS radical scavenging assay

The ABTS assay was used to measure the free radical scavenging capabilities of the precursor hHb and PDA-coated hHbs (PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15) (Fig. 6B and Table 1). This method assesses the reduction of ABTS^{•+} radicals, which is detected as a decrease in the absorbance at 734 nm, providing a measure of antioxidant activity. The ABTS^{•+} radical scavenging activity of 1 µg of hHb, PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15 was quantified and reported as the percentage of free radicals scavenged. The ABTS scavenging activity for hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 was $1.6 \pm 0.1 \%$, $2.6 \pm 0.1 \%$, $2.9 \pm 0.1 \%$, and $2.5 \pm 0.1 \%$, respectively. The results show that all three PDA-coated hHbs displayed significantly higher radical scavenging activity compared to the precursor hHb ($p < 0.001$). PDA NPs, without hHb, displayed a scavenging activity of $3.8 \pm 0.3 \%$, confirming the increased radical scavenging activity of PDA-coated hHbs was due to the PDA coating. Interestingly, PDA-hHb-13 exhibited a higher radical scavenging activity ($p < 0.05$) compared to PDA-hHb-1 and PDA-hHb-15, which had similar activities ($p > 0.05$). These findings highlight that the application of a PDA coating enhances the antioxidant capability of hHb by increasing its capacity to neutralize cytotoxic free radicals.

3.12. Hydrogen peroxide mediated oxidation

In this experiment, ferryl Hb was produced by incubating oxyHb with hydrogen peroxide (H₂O₂) at a heme:H₂O₂ molar ratio of 1:10 for five minutes at room temperature. The reaction was terminated by adding excess catalase. The levels of ferryl Hb were indirectly assessed by quantifying sulfhemoglobin (sulfHb) formation, which was identified by an absorption peak at 620 nm following the reaction with sodium sulfide (Na₂S) (Fig. 7A and Table 1). The results demonstrate a notable change in sulfHb concentration between the precursor hHb and the PDA-coated hHbs (PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15), underscoring the influence of the PDA coatings on H₂O₂ mediated oxidation. The precursor hHb exhibited a sulfHb concentration of $0.3 \pm 0.0 \mu\text{M}$, indicating limited formation of ferryl Hb under oxidative conditions. PDA-hHb-1 showed a higher concentration of sulfHb, compared to hHb ($p < 0.05$) but was significantly lower than both PDA-hHb-13 and PDA-hHb-15 ($p < 0.001$). Conversely, PDA-hHb-13 and PDA-hHb-15 displayed sulfHb concentrations of $6.6 \pm 0.4 \mu\text{M}$ and $6.0 \pm 0.1 \mu\text{M}$, significantly higher than both hHb and PDA-hHb-1 ($p < 0.001$), suggesting a pronounced increase in oxidative reactivity and ferryl Hb formation. PDA-hHb-13 and PDA-hHb-15 formed similar amounts of sulfHb with no statistical significance between each other ($p > 0.05$).

3.13. Catalase activity assay

Catalase activity was evaluated by observing the breakdown of H₂O₂ in the presence of the precursor hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 using UV-visible spectroscopy at 240 nm (Fig. 7B and Table 1). A standard curve was utilized to quantify the catalase content in each sample. The results indicate that the precursor hHb exhibits the highest catalase activity at $20.3 \pm 0.7 \text{ units/mL}$ ($p < 0.001$) of all the groups, while the PDA-coated variants show a marked reduction compared to hHb. Amongst the PDA-hHbs, PDA-hHb-1 showed the highest catalase activity at $12.2 \pm 0.8 \text{ units/mL}$ ($p < 0.001$), roughly 60 % of the activity of the precursor hHb. PDA-hHb-13 displayed an activity of $4.6 \pm$

0.2 units/mL, representing roughly 23 % of the activity of the precursor hHb, whereas PDA-hHb-15 had a catalase activity of 3.1 ± 0.04 units/mL, which was slightly lower than that of PDA-hHb-13 ($p < 0.048$), and approximately 15 % of that of the precursor hHb. This result is consistent with the retention of catalase, which has a molecular weight of approximately 240 kDa, during the TFF purification of precursor hHb using a 50 kDa TFF module [40]. The enzymatic activity of the catalase in PDA coated variants could be potentially affected by the PDA coating process, resulting in decreased activity. These results suggest that PDA coating affects catalase activity in a manner dependent on the coating conditions, with increasing PDA modification leading to greater enzymatic inhibition.

3.14. Circular dichroism spectroscopy

The secondary and tertiary structures of the precursor hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 was assessed using CD spectroscopy in the far-UV (190–260 nm) and near-UV (250–500 nm) regions (Fig. 8A and B), respectively. In the far-UV region, all samples demonstrated molar ellipticity minima at 208 nm and 222 nm, indicative of the alpha helical content characteristic of the hHb's secondary structure. The molar ellipticity for hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 at 208 nm was $-10.2 \times 10^6 \pm 1.0 \times 10^5$ deg. cm² mol⁻¹, $-9.8 \times 10^6 \pm 9.4 \times 10^5$ deg. cm² mol⁻¹, $-9.3 \times 10^6 \pm 5.4 \times 10^5$ deg. cm² mol⁻¹, and $-8.7 \times 10^6 \pm 0.3 \times 10^5$ deg. cm² mol⁻¹ respectively, while at 222 nm was $-12.8 \times 10^6 \pm 0.6 \times 10^5$ deg. cm² mol⁻¹, $-12.1 \times 10^6 \pm 11.0 \times 10^5$ deg. cm² mol⁻¹, $-11.4 \times 10^6 \pm 6.1 \times 10^5$ deg. cm² mol⁻¹, and $-10.7 \times 10^6 \pm 1.0 \times 10^5$ deg. cm² mol⁻¹ respectively. A reduction in molar ellipticity was observed for PDA-hHb-15 compared to the precursor hHb ($p < 0.05$), suggesting a reduction in alpha-helical content. No change in molar ellipticity was observed for PDA-hHb-1 and PDA-hHb-13 compared to the precursor hHb. These changes indicate partial disruption of alpha helical regions due to PDA modification. At 421 nm, corresponding to the heme pocket (near-UV), the molar ellipticity did not show statistically significant changes between the precursor hHb ($2.9 \times 10^5 \pm 6.6 \times 10^4$ deg. cm² mol⁻¹), PDA-hHb-1 ($2.6 \times 10^5 \pm 7.0 \times 10^4$ deg. cm² mol⁻¹), PDA-hHb-13 ($3.0 \times 10^5 \pm 1.1 \times 10^4$ deg. cm² mol⁻¹), and PDA-hHb-15 ($2.8 \times 10^5 \pm 2.0 \times 10^4$ deg. cm² mol⁻¹) ($p > 0.05$). This indicates that the heme pocket remained unaltered, suggesting a more ordered or tightly bound heme configuration.

3.15. Thermal stability

The thermal stability of hHb and its PDA-coated hHb variants was assessed using CD spectroscopy to determine their melting temperatures (T_m) (Fig. 8C). Native hHb exhibited the highest thermal stability with respect to all PDA-hHb variants, with a T_m of 65.88 ± 0.17 °C ($p < 0.001$). In contrast, PDA-coated Hb samples showed a slight reduction in thermal stability, consistent with previous reports in the literature that describe similar effects of PDA coatings on Hb [46]. Amongst the variants, PDA-hHb-13 had the lowest T_m at 59.33 ± 0.15 °C, while PDA-hHb-1 and PDA-hHb-15 displayed a marginally higher T_m of 61.0 ± 0.6 °C and 60.57 ± 0.13 °C. This reduction in T_m for PDA-hHb variants compared to hHb suggests that the PDA coating may affect the protein's conformational stability, potentially due to interactions between the PDA layer and Hb that compromise its' thermal resilience. The slight but significant differences between PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15

($p < 0.05$) implies that the extent or specific conditions of the PDA coating process might influence the degree of thermal destabilization or stabilization.

4. Discussion

While there have been many studies investigating the PDA coating of Hb and HBOCs, to the best of our knowledge, we are the first to implement CO-binding of hHb before PDA coating to reduce oxidation of ferrous Hb, and to synthesize PDA NPs to limit the interaction of dopamine with the heme pocket. In this work, we have synthesized PDA-coated hHb by two new methods (PDA-hHb-13 and PDA-hHb-15) and the method described in literature (PDA-hHb-1). We have also calculated the mass of dopamine incorporated in the PDA coating using a mass balance approach to ensure the three groups being evaluated (PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15) have similar levels of dopamine incorporation, in order to compare their properties. Using DLS we found that PDA-hHb-1 and PDA-hHb-13 did not show a significant increase in their hydrodynamic diameter compared to the precursor hHb (~5 nm), while PDA-hHb-15 exhibited a marked increase in size (~385 nm) owing to the PDA NPs (~110 nm) formed in the first step of its synthesis process. The larger size of PDA-hHb-15 implies a potentially faster clearance via the reticuloendothelial system (RES) in the liver and spleen, and could result in a shorter circulatory half-life [48]. PDA-hHb-15 also displayed a higher polydispersity index compared to hHb, while the polydispersity for PDA-hHb-1 and PDA-hHb-13 was similar to hHb.

Previous studies have concluded that PDA coatings reduce metHb formation through increased FRAP activity [36]. However, while these assays assess the capacity of PDA-Hb to reduce external Fe^{3+} ions, they do not provide insight into the auto-oxidation kinetics or the inherent metHb levels of PDA-coated Hb, where Fe^{3+} is integrated within the heme moiety of hHb. Therefore, further investigation is required to elucidate the direct impact of PDA coatings on the intrinsic oxidative stability of Hb. In this study, the traditional PDA coating method at basic pH (PDA-hHb-1) resulted in a significant increase in metHb levels (~13 %). To mitigate this, we developed PDA-hHb-13 where Hb was CO-locked prior to PDA coating and subsequently CO-unlocked after purification. PDA-hHb-13 exhibited reduced metHb level (~5 %) post unlocking but displayed a significantly higher auto-oxidation rate compared to the precursor hHb. Molecular docking experiments by Nadimifar et al., 2023 suggest that dopamine interacts with Hb near the heme pocket [46]. We hypothesize that this interaction may facilitate the entry of water molecules to the heme pocket, driven by dopamine's hydrophilicity, thereby facilitating metHb formation. To prevent dopamine binding near the heme pocket, we developed PDA-hHb-15 by first polymerizing dopamine into PDA nanoparticles before coating it with hHb. The larger molecular size 110 ± 23 nm of the PDA nanoparticle compared to dopamine likely restricts its penetration into the globin chains, reducing its interaction near the heme pocket. Auto-oxidation kinetics revealed that PDA-hHb-15 exhibited a higher auto-oxidation rate constant than the precursor hHb, but was nearly half that of PDA-hHb-13. Moreover, PDA-hHb-15 had metHb levels of ~5 % post-unlocking, comparable to PDA-hHb-13. We also investigated the auto-oxidation kinetics of PDA-hHb-1, which demonstrated an auto-oxidation rate constant similar to PDA-hHb-13. This supports our hypothesis that the interaction of dopamine near the heme pocket may promote metHb formation.

PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15 demonstrated significantly enhanced ferric reducing (FRAP) activity and ABTS radical scavenging activity compared to the precursor hHb, indicating successful incorporation of antioxidant properties to the HBOC. The sulfHb assay was performed to evaluate the protection from H₂O₂-mediated oxidation to Fe⁴⁺, where all three PDA-coated Hbs showed significantly higher Fe⁴⁺ formation compared to the precursor hHb. This can be explained by the presence of catalase in the precursor hHb as shown by our previous study [40]. The catalase activity assay confirmed a significantly higher catalase activity for the precursor hHb compared to the three PDA-coated hHbs (PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15). Interestingly, PDA-hHb-1 exhibited lower sulfHb formation compared to PDA-hHb-13 and PDA-hHb-15, which could be explained by its higher catalase activity compared to PDA-hHb-13 and PDA-hHb-15.

PDA-hHb-13 exhibited a significantly higher P₅₀ compared to hHb, whereas PDA-hHb-15 did not show any significant change in P₅₀, compared to hHb. These findings are in agreement with the study by Nadimifar et al., 2023 which demonstrated that PDA coating increases the P₅₀ due to dopamine binding in the central water cavity of Hb, a key region responsible for allosteric regulation. During the synthesis of PDA-hHb-13, the simultaneous addition of dopamine and hHb, likely facilitated dopamine binding in the central water cavity, leading to an increased P₅₀. In contrast, PDA-hHb-15 involves the polymerization of dopamine into PDA nanoparticles, prior to its' coating with hHb. Due to its larger molecular size (110 nm), PDA nanoparticles are less likely to penetrate the central water cavity, instead interacting primarily at the hHb surface. Consequently, PDA-hHb-15 did not exhibit a significant change in P₅₀ compared to hHb. These observations further support the findings of Nadimifar et al., 2023, who reported that non-linearly polymerized dopamine primarily engages in surface interactions with Hb and without deeper penetration into the protein structure.

It is generally observed that the higher the P₅₀, the higher the oxygen offloading rate [40,49]. We observe this correlation between the PDA-hHbs (PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15). The order for P₅₀ is PDA-hHb-13 > PDA-hHb-15 > PDA-hHb-1, and the order for the oxygen offloading rate is PDA-hHb-13 > PDA-hHb-15 = PDA-hHb-1. However, when compared with the precursor hHb, the observed order for PDA-hHbs seems to be lower than expected. We think this decrease could be due to diffusional resistance by the PDA layer, as other surface modifications of Hb such as PEGylation and encapsulation in resealed RBC ghosts have also shown a decrease in oxygen offloading kinetics [49,50]. Although the oxygen offloading rate for PDA-hHb-15 is ~20–25 % lower than that of hHb, it is still higher than that of native RBCs, measured around 8.6 s⁻¹ [51]. We therefore believe that the offloading rate for PDA-hHb-15 is adequate for oxygen delivery, under conditions of high oxygen demand.

The effect of the PDA coating on the binding of the PDA-hHb variants to haptoglobin (Hp) was also studied. All three PDA-coated hHbs (PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15) had similar Hp binding rates compared to the precursor hHb. This indicates that the PDA-coating process does not significantly alter the interaction of the proteins with Hp and that PDA-coated hHbs should have similar macrophage uptake rates through the CD163-mediated endocytosis pathway.

CD spectroscopy in the far-UV region revealed a reduction in molar ellipticity at the α -helical minima (208 nm and 222 nm) for PDA-hHb-15 compared to the precursor hHb, indicating a progressive loss of α -helical content. This trend is consistent with the previously reported literature, where PDA coating caused a decrease in α -helical structure and an increase in β -sheets, β -turns, and unordered structures. Additionally, no alterations in the heme pocket structure were observed at 421 nm for all the PDA-coated hHbs (PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15). Thermal stability analysis revealed that the precursor Hb exhibited the highest melting temperature (T_m), indicative of its superior stability. The PDA coatings caused a reduction in T_m for PDA-hHb-1, PDA-hHb-13 and PDA-hHb-15 compared to the precursor hHb. This trend is consistent with the previously reported literature [46], where T_m values decreased for PDA-coated Hb, signifying reduced stability due to PDA interactions with the globins. Overall, the current study closely follows the trends reported in the literature [46], reinforcing the idea that PDA coatings simultaneously modify structural elements of Hb, particularly by modifying its secondary structure (α -helices). These modifications may disrupt hydrogen bonding and electrostatic interactions, potentially leading to partial protein unfolding.

Compared to the commercial HBOCs - HemeAssist and MP4, PDA-hHb-1 and PDA-hHb-13 had similar auto-oxidation rate constants, while PDA-hHb-15 showed a reduced auto-oxidation rate constant, indicating better stability and functionality. PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 also demonstrated enhanced catalase activity and lower oxidation to sulfHb compared to HemeAssist and MP4, highlighting their antioxidant protection against H_2O_2 . The three PDA-hHbs (PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15) exhibited lower oxygen offloading rates compared to the two commercial HBOCs, which could be undesirable when the goal is fast oxygen delivery to hypoxic tissues. The PDA-hHbs have similar haptoglobin binding rates compared to hHb, but higher than the commercial HBOCs, implying a potentially shorter circulatory life for PDA-hHbs. The P_{50} and cooperativity for the PDA-hHbs are lower compared to HemeAssist, which could be undesirable for fast oxygen release. Thus, PDA-hHbs demonstrate enhanced antioxidant properties compared to the commercial HBOCs, but further optimization is needed to compete with properties of oxygen transport and haptoglobin binding.

5. Conclusion

The development of HBOCs has been largely hindered by oxidative toxicity stemming from the heme group of Hb. While PDA coating of HBOCs has been previously explored as a strategy to mitigate these issues, a comprehensive biophysical assessment of its effects on HBOC auto-oxidation kinetics, ferryl species generation, and Hp binding has not been conducted until this current study. In this study, we found that PDA-hHb synthesized from previously reported methods (PDA-hHb-1) yielded significantly higher metHb content (~13 %). To address this, we synthesized PDA-hHb-13 where hHb was bound to CO which reduced metHb levels to ~5 %. PDA-hHb-1 and PDA-hHb-13 both had high auto-oxidation rates, potentially due to the interaction of dopamine with the heme pocket. We then synthesized PDA-hHb-15 where PDA NPs were first formed and then bound to hHb, in an effort to reduce the dopamineheme interaction. PDA-hHb-15 demonstrated metHb levels of (~5 %) and lower auto-oxidation rates – half that of PDA-hHb-1 and PDA-hHb-13.

Antioxidant assays (FRAP and ABTS) confirmed enhanced antioxidant activity across all PDA-hHbs, though sulfHb formation assays suggested increased susceptibility to H₂O₂-mediated oxidation, likely due to reduced catalase activity upon PDA coating. Oxygen transport properties, although slightly altered upon PDA coating, are still suitable for HBOC applications. PDA-hHb-13 exhibited a lower oxygen affinity, which is desirable for oxygen release. PDA-hHb-15 exhibited a lower oxygen offloading rate compared to hHb but was still higher than that of RBCs. PDA-hHb-15 also exhibited a considerably higher size (~385 nm), implying a potentially faster clearance via the RES.

Overall, this work provides significant advances in PDA coating of HBOCs by introducing CO binding of Hb, and PDA coating using pre-formed PDA NPs that reduce methHb formation and rate of auto-oxidation, respectively. Yet, further optimization, such as modulating particle size, is needed to improve the in vivo performance of PDA-hHb as an HBOC.

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Data availability

Data will be made available on request.

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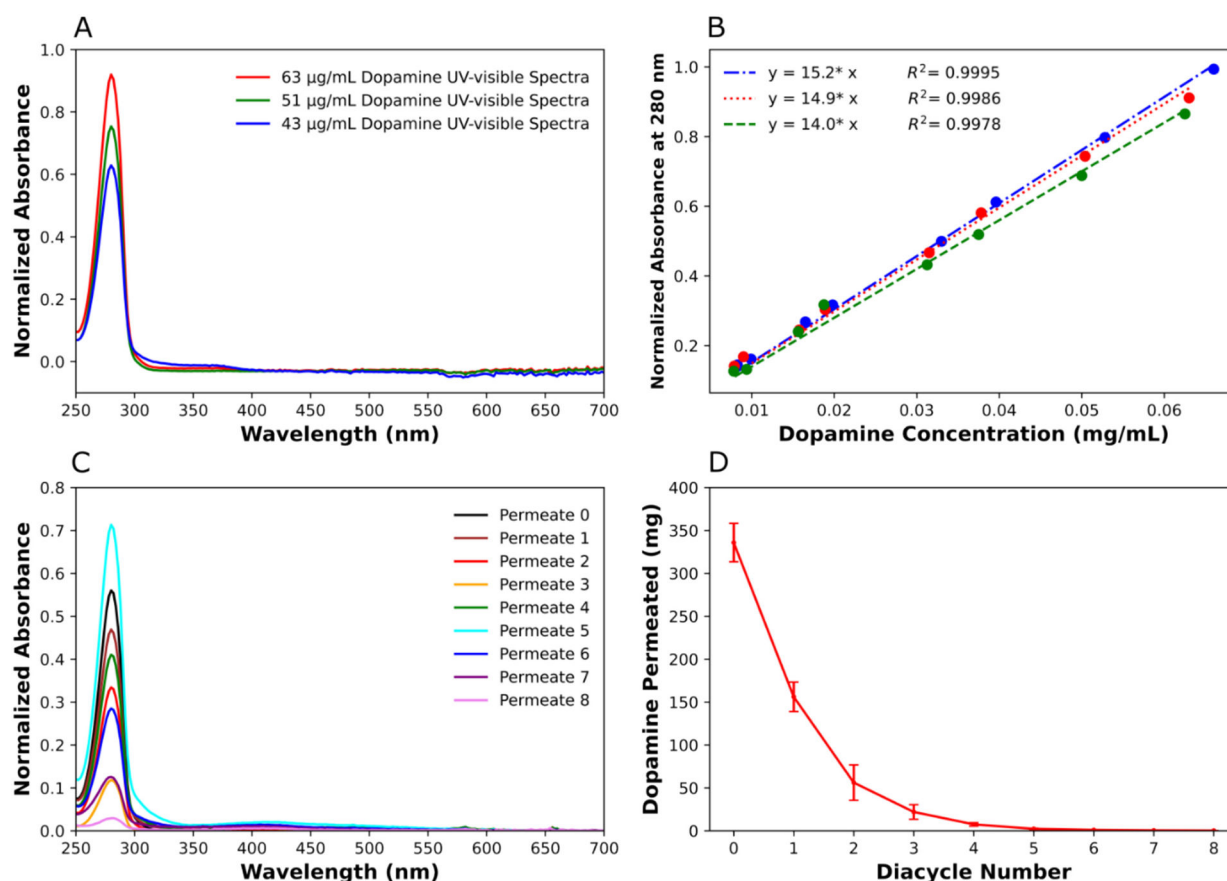
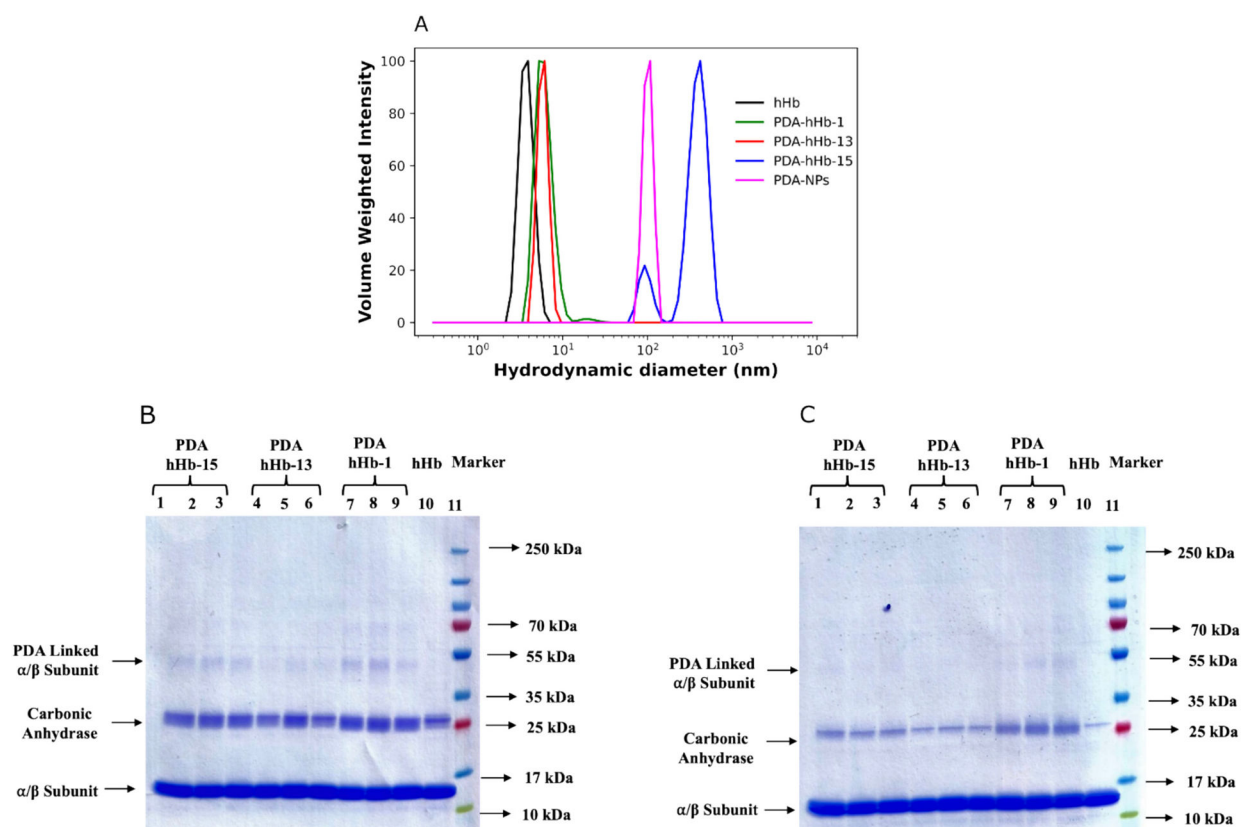


Fig. 1.

(A) UV–visible absorption spectra of dopamine. (B) Dopamine calibration curve. (C) UV–visible absorption spectra of TFF permeates at each diacycle during TFF purification. (D) Mass of dopamine permeated during each diacycle of PDA-hHb-15 purification.

**Fig. 2.**

(A) Representative DLS analysis for hHb, PDA-hHb-1, PDA-hHb-13, PDA-hHb-15, and PDA-NPs. SDS-PAGE of the precursor hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 under (B) non-denaturing conditions (absence of DTT), and (C) denaturing conditions (presence of DTT).

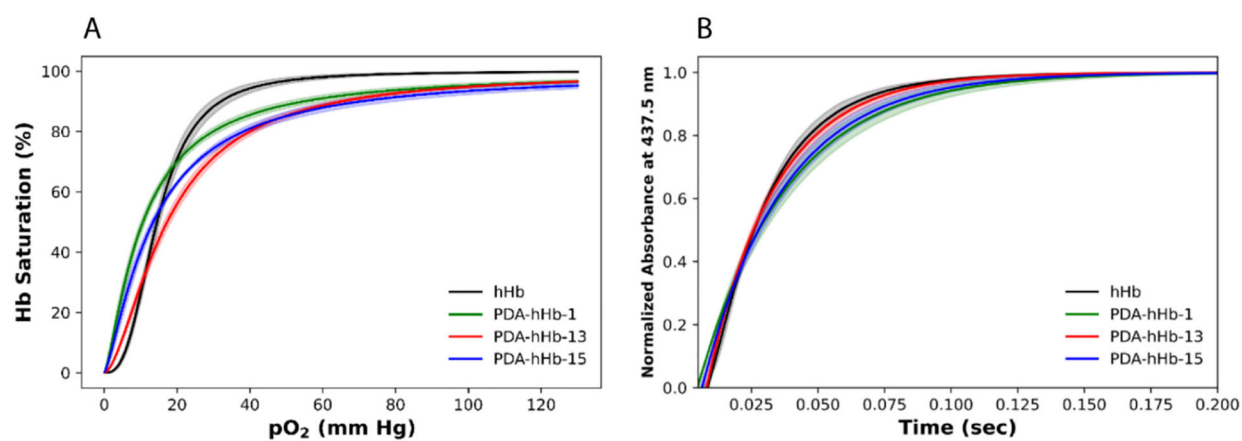


Fig. 3. (A) Oxygen equilibrium curves at pH 7.4 and 37°C and (B) Oxygen offloading kinetics of hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15.

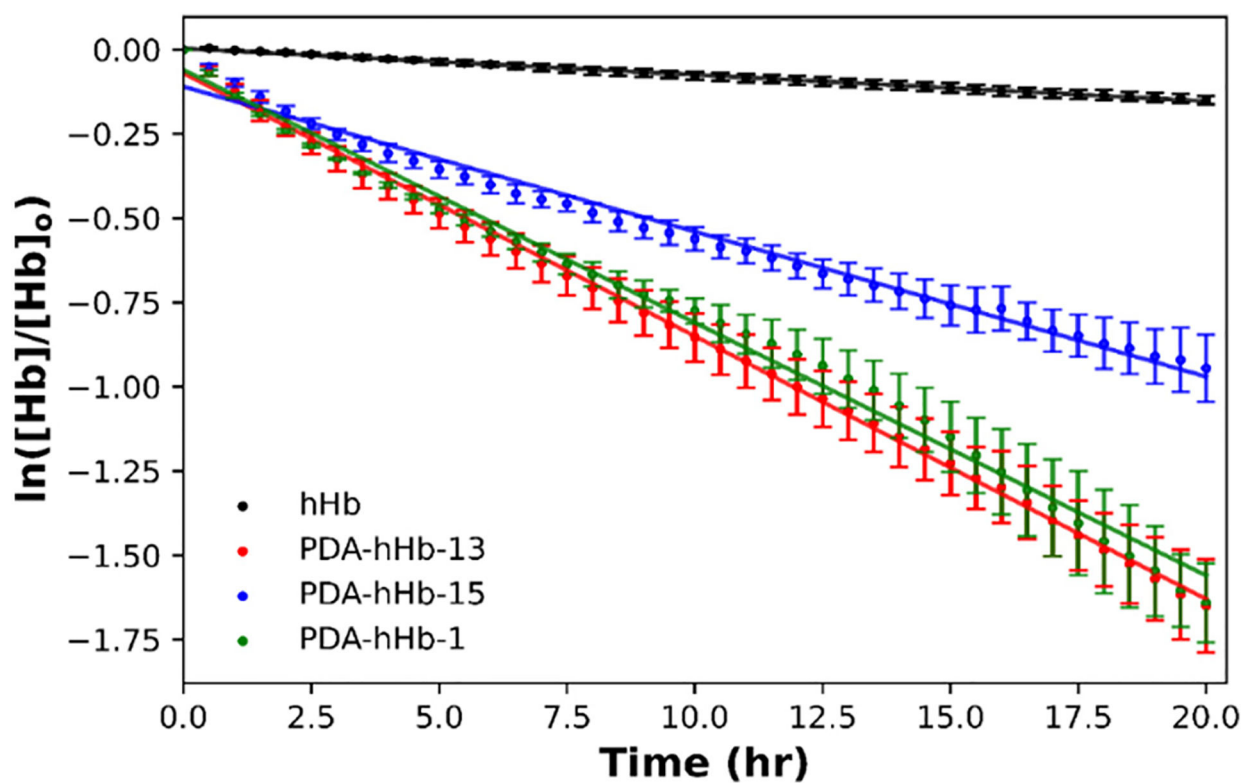


Fig. 4.
Auto-oxidation kinetics of hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15.

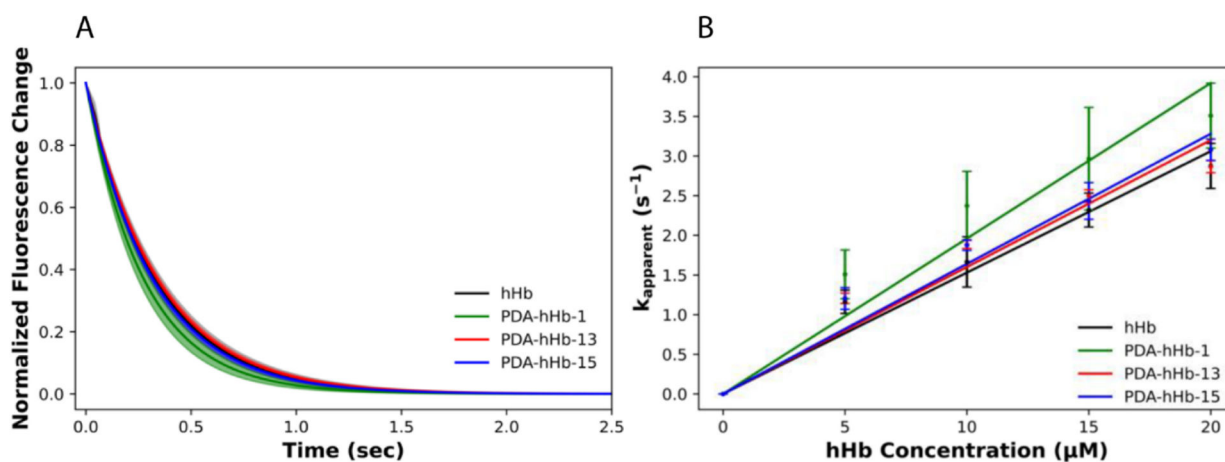


Fig. 5.

(A) Representative average kinetic traces for hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15 binding to haptoglobin (Hp) at 0.25 μM . (B) The apparent pseudo-first order rate constant for Hp binding to hHb/PDA-hHb was plotted as a function of protein concentration to regress the second order rate constant for Hp binding.

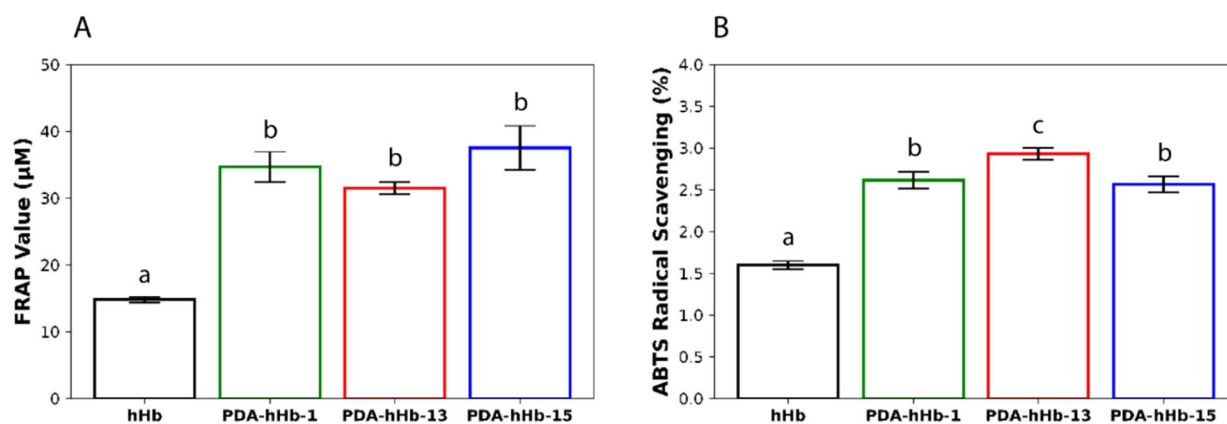


Fig. 6. (A) FRAP activity quantification of hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15. (B) ABTS radical scavenging activity quantification of hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15. Tukey's means comparison results are depicted using lower-case letters where groups not connected by the same letters are statistically different ($p < 0.05$).

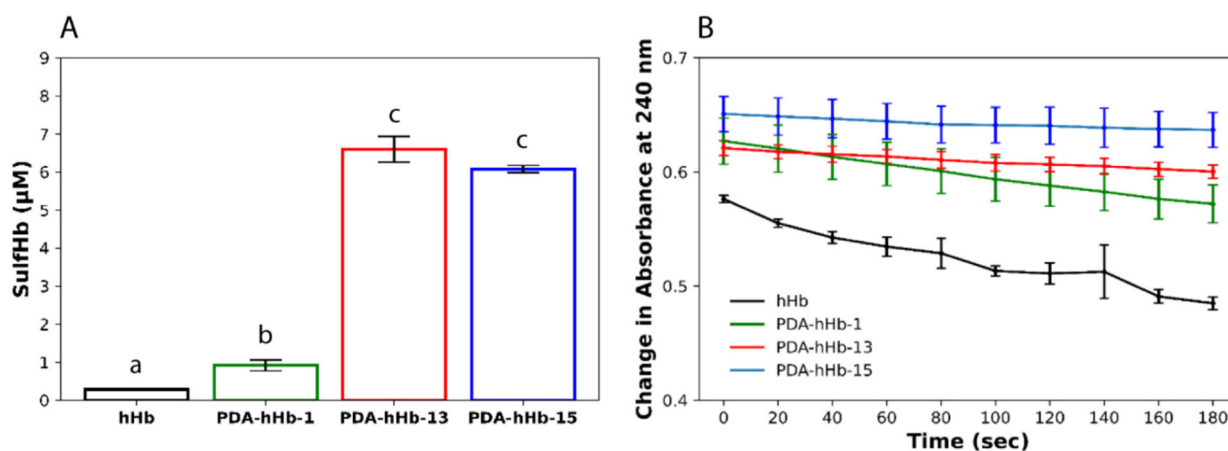


Fig. 7.

(A) Quantification of Sulfheme formation of hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15. (B) Catalase activity of hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15.

Tukey's means comparison results are depicted using lower-case letters where groups not connected by the same letters are statistically different ($p < 0.05$).

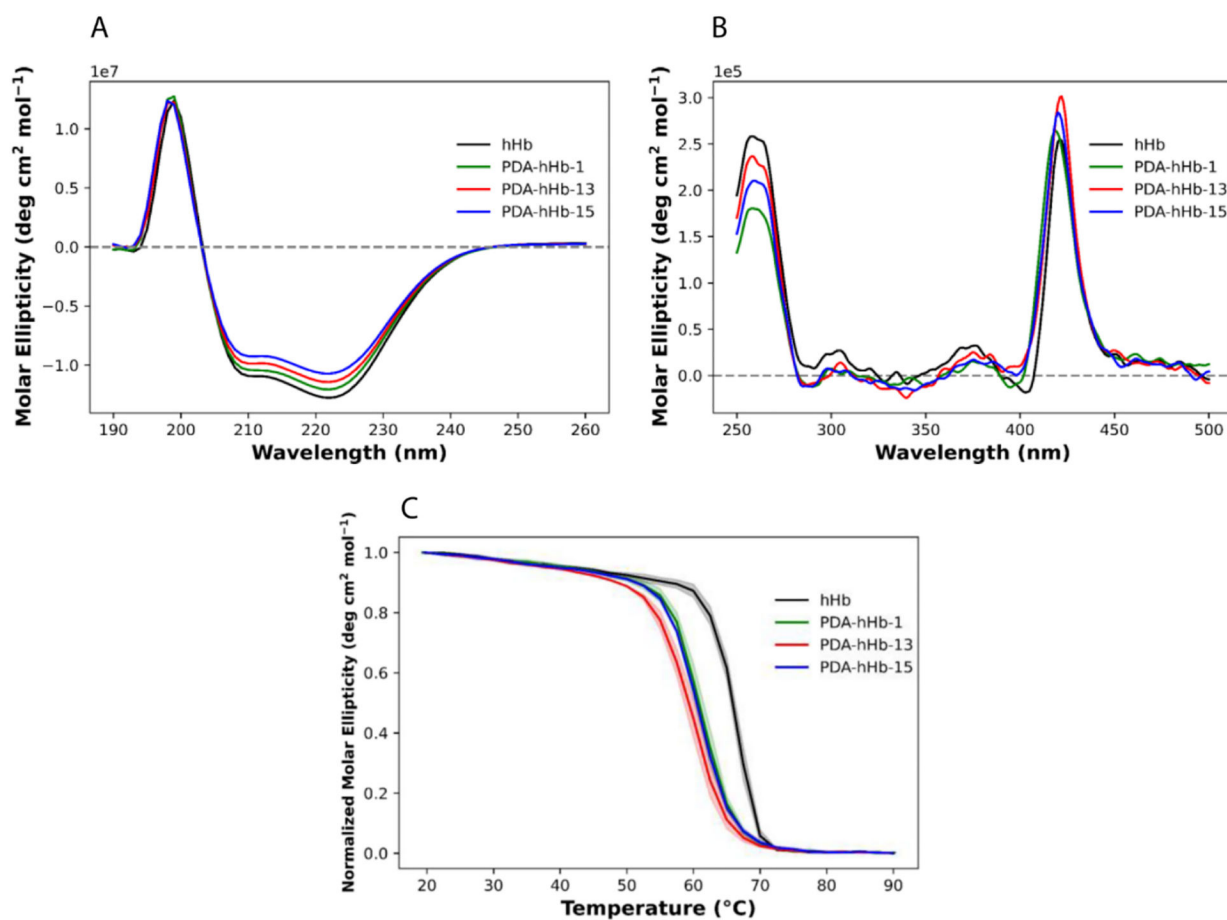
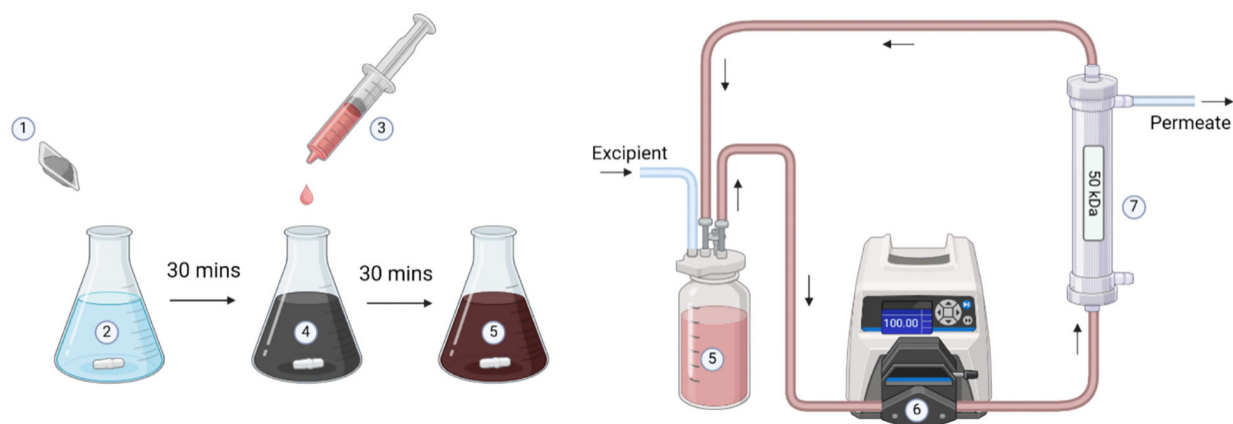


Fig. 8. (A) Far UV and (B) Near UV circular dichroism spectra of hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15. (C) Thermal stability analysis of hHb, PDA-hHb-1, PDA-hHb-13, and PDA-hHb-15.



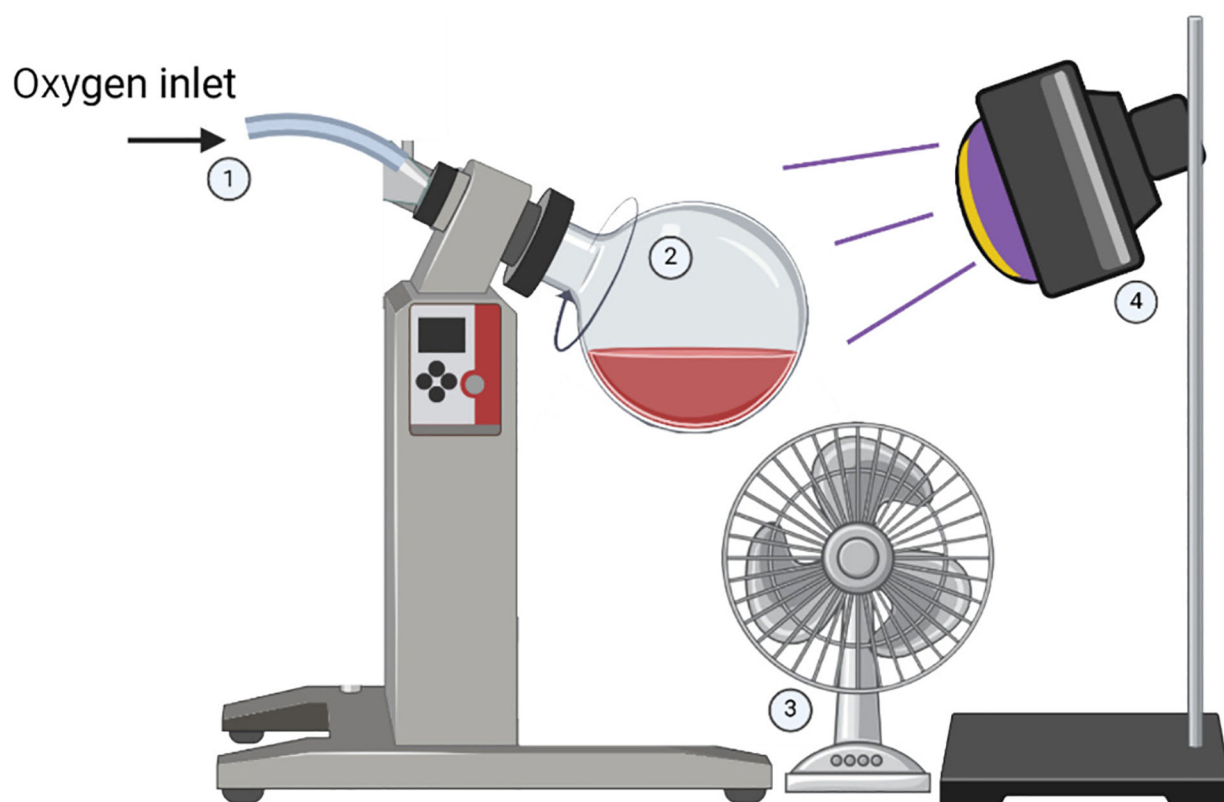
PDA-hHb-15 Synthesis and Purification

Components:

- | | |
|---------------------------|-----------------------------------|
| 1. Dopamine-HCl | 5. PDA-hHb-15 |
| 2. Tris Buffer (pH = 8.5) | 6. Peristaltic Pump |
| 3. hHbCO | 7. 50 kDa TFF Hollow Fiber Module |
| 4. PDA-NPs | |

Scheme 1.

Schematic of PDA-hHb-15 synthesis and purification via tangential flow filtration.



Apparatus for CO-unlocking:

1. Oxygen Supply
2. Round-bottom Flask
3. Cooling Fan
4. UV Lamp

Scheme 2.

Schematic for CO-unlocking of PDA-hHb-13 and PDA-hHb-15.

Table 1

Comprehensive biophysical and biochemical properties of hHb, PDA-hHb-1 (literature method), PDA-hHb-13 (PDA coating of hHbCO) and PDA-hHb-15 (coating using pre-formed PDA NPs) compared to the commercial HBOCs HemAssist and MP4.

Biophysical Properties	hHb	PDA-hHb-1	PDA-hHb-13	PDA-hHb-15	HemAssist [45]	MP4 [45]
metHb Level (%)	1.0 ± 0.1 ^{b,c,d}	13.7 ± 1.5 ^{a,c,d}	4.4 ± 0.5 ^{a,b}	5.6 ± 0.4 ^{a,b}	NA	NA
Dopamine in PDA Coating (mg dopamine / g Hb)	N/A	42.7 ± 19.6	34.3 ± 7.1	61.3 ± 27.7	NA	NA
k _{auto-ox} (h ⁻¹)	0.008 ± 0.001 ^{b,c,d}	0.075 ± 0.009 ^{a,d}	0.078 ± 0.006 ^{a,d}	0.043 ± 0.004 ^{a,b,c}	0.081 ± 0.21	0.070 ± 0.002
SulfHb (μM) 1:10 (Heme: H ₂ O ₂)	0.3 ± 0.0 ^{b,c,d}	0.9 ± 0.2 ^{a,c,d}	6.6 ± 0.4 ^{a,b}	6.1 ± 0.1 ^{a,b}	18.8 ± 0.21 *	6.43 ± 0.34 *
Catalase activity (units/mL)	20.3 ± 0.7 ^{b,c,d}	12.2 ± 0.8 ^{a,c,d}	4.6 ± 0.2 ^{a,b,d}	3.1 ± 0.0 ^{a,b,c}	0.09	0.41
P ₅₀ (mm Hg)	14.5 ± 0.7 ^{b,c}	10.6 ± 0.6 ^{a,c,d}	17.2 ± 0.9 ^{a,b,d}	13.4 ± 0.6 ^{b,c}	31.10 ± 0.27	8.18 ± 0.12
n	2.8 ± 0.2 ^{b,c,d}	1.3 ± 0.1 ^a	1.6 ± 0.02 ^a	1.3 ± 0.1 ^a	2.46 ± 0.10	1.56 ± 0.01
k _{O₂, off} (s ⁻¹)	41.1 ± 0.8 ^{b,d}	30.2 ± 3.2 ^{a,c}	41.3 ± 1.3 ^{b,d}	32.9 ± 3.4 ^{a,c}	117.1 ± 0.4	67.2 ± 0.2
k _{Hb-Hb} (μM ⁻¹ s ⁻¹)	0.15 ± 0.02	0.20 ± 0.03	0.16 ± 0.00	0.16 ± 0.01	0.003 ± 0.000	0.030 ± 0.000
FRAP (μM) [^]	14.8 ± 0.5 ^{b,c,d}	34.7 ± 2.7 ^a	31.5 ± 1.1 ^a	28.6 ± 1.4 ^a	—	—
ABTS (%) [^]	1.6 ± 0.1 ^{b,c,d}	2.6 ± 0.1 ^{a,c}	2.9 ± 0.1 ^{a,b,d}	2.6 ± 0.1 ^{a,c}	—	—
Dia (nm) (DLS)	3.7 ± 0.3 ^d	6.5 ± 1.3 ^d	6.0 ± 1.0 ^d	385.0 ± 54.0 ^{a,b,c}	—	—
PDI (DLS)	0.05 ± 0.02 ^d	0.07 ± 0.02 ^d	0.02 ± 0.0 ^d	1.25 ± 0.05 ^a	—	—
T _m (°C)	65.9 ± 0.2 ^{b,c,d}	61.0 ± 0.6 ^{a,c}	59.3 ± 0.2 ^{a,b}	60.6 ± 0.1 ^a	—	—

Significantly different compared to ^a hHb, ^b PDA-hHb-1, ^c PDA-hHb-13, ^d PDA-hHb-15 ($p < 0.05$).

* SulfHb formation for 1:1 Heme: H₂O₂.

[^] Represented per μg of protein.