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Biophysical and Biochemical Characterization of High Molecular Weight Co-Polymerized Human Hemoglobin and Albumin as a Potential Hemoglobin-Based Oxygen Carrier

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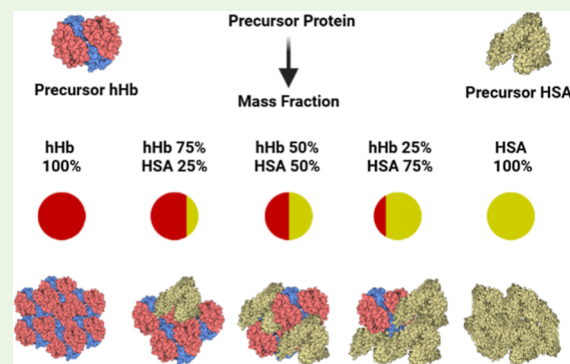
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ABSTRACT: Human hemoglobin (hHb) in the tense (T) quaternary state was copolymerized with human serum albumin (HSA) at various hHb:HSA mass fractions to form polymerized hHb-HSA Poly(hHb:HSA) conjugates as a potential next-generation hemoglobin-based oxygen carrier (HBOC). These conjugates were evaluated for molecular weight (M_W), hydrodynamic size, oxygen transport characteristics, heme and oxidative stability, as well as hemorheological and colloid osmotic pressure (COP) properties. Among the variants, Poly(hHb₇₅:HSA₂₅) achieved a high M_W (2024 ± 262 kDa), hydrodynamic diameter (29.6 ± 2.3 nm), yield ($39 \pm 1\%$), and batch mass (11.8 ± 0.2 g), closely matching PolyhHb₁₀₀. In comparison, Poly(hHb₅₀:HSA₅₀) exhibited a lower M_W (875 ± 84 kDa) and diameter (21.5 ± 1.9 nm), with a reduced yield ($26 \pm 4\%$) and batch mass (7.7 ± 1.3 g). Both formulations demonstrated rapid oxygen offloading (63.1 ± 0.5 and 59.0 ± 1.4 s⁻¹) and low oxygen affinity ($P_{50} = 49.04 \pm 0.95$ and 41.79 ± 0.81 mmHg), indicating effective oxygen delivery under moderate oxygen tensions. Although polymerization modestly elevated the auto-oxidation rate compared to the precursor hHb, the oxidative stability remained comparable between Poly(hHb₇₅:HSA₂₅) and Poly(hHb₅₀:HSA₅₀), suggesting that HSA incorporation does not significantly impact the rate of auto-oxidation. Both Poly(hHb₇₅:HSA₂₅) and Poly(hHb₅₀:HSA₅₀) reduced haptoglobin binding (0.005 and 0.004 μM^{-1} s⁻¹) and heme release rates, reflecting enhanced heme retention and reduced oxidative risk. Both Poly(hHb₇₅:HSA₂₅) and Poly(hHb₅₀:HSA₅₀) exhibited similar zeta potentials (-23.2 ± 1.3 mV and -27.0 ± 1.7 mV respectively). Structural analyses confirmed the preserved α -helical content, thermal stability (69.5 – 70.9 °C), and retained intrinsic catalase activity of the two variants. Hemorheological and COP analyses further revealed that both Poly(hHb₇₅:HSA₂₅) and Poly(hHb₅₀:HSA₅₀) exhibited low COP, were hyperviscous solutions with shear-thinning behavior, and exhibited reversible red blood cell (RBC) aggregation at low shear rates. Therefore, both T-state Poly(hHb₇₅:HSA₂₅) and Poly(hHb₅₀:HSA₅₀) offer an optimal balance of oxygen delivery, oxidative resilience, manufacturability, shear-thinning behavior, making them strong candidates for further HBOC development.

KEYWORDS: polymerized human hemoglobin, polymerized human serum albumin, co-polymerized hemoglobin and albumin, hemoglobin-based oxygen carrier, oxygen therapeutic, red blood cell substitute



1. INTRODUCTION

Blood is essential for sustaining human life by transporting oxygen from the lungs to tissues and removing carbon dioxide. A consistent blood supply is vital for treating chronic diseases, trauma, hemolytic disorders, and supporting surgical procedures.^{1,2} However, red blood cell (RBC) transfusions face global donor shortages, health and religious limitations, disease transmission risks, immune reactions, blood type incompatibility, and storage constraints.^{3–6} These issues are exacerbated during emergency scenarios such as natural disasters.⁷ To address these challenges, artificial oxygen carriers (AOCs), notably hemoglobin-based oxygen carriers (HBOCs) and

perfluorocarbon (PFC) based carriers are being developed as substitutes to replicate the oxygen carrying role of RBCs.^{8–10}

HBOCs capitalize on hemoglobin's (Hb) natural oxygen-carrying ability and are derived from outdated human or bovine RBCs or produced recombinantly.¹¹ Outside of the

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RBC, however, tetrameric Hb ($\alpha_2\beta_2$) dissociates into $\alpha\beta$ dimers and α/β monomers, leading to renal clearance and toxicity. Cell-free human Hb also lacks the key allosteric modulator 2,3-DPG and antioxidant RBC enzymes, thus reducing oxygen delivery efficiency and increasing oxidative stress. Additionally, Hb scavenges nitric oxide (NO), a vasodilator, causing vasoconstriction and hypertension.^{12,13} Therefore, various modifications such as polymerization, PEGylation, encapsulation, and recombinant engineering have been employed to address these drawbacks.^{14–17}

Cross-linking Hb with agents such as diaspirin or raffinose stabilizes Hb by preventing tetramer dissociation into renally cleared dimers. Diaspirin cross-linked Hb (DCLHb or HemAssist), developed by Baxter International and the U.S. Army, linked α -subunits (Lys99 $\alpha 1$ and Lys99 $\alpha 2$) using bis(3,5-dibromosalicyl) fumarate (DCLHb, M_w 64 kDa, P_{50} $\sim$ 30 mmHg), but was discontinued in Phase III clinical trials due to increased mortality.^{18–21} Optro, from Somatogen Inc., used a glycine-bridged α – α linkage in Hb Presbyterian (β N108 K) expressed in *E. coli*, but was halted in Phase I/II elective surgery trials due to hypertensive effects.^{17,22,23} PEGylated Hb variants aimed to enhance circulatory stability. MP4 (Hemospan), a high-affinity PEGylated Hb (M_w 95 kDa, P_{50} = 4–8 mmHg) developed by Sangart Inc. using site-specific thiolation for elective surgery was stopped due to funding issues.^{24,25} Sanguinate, a nonsite-specific PEGylated bovine Hb (M_w 120 kDa, P_{50} $\sim$ 11 mmHg) by Prolong Pharmaceuticals entered Phase I/Ib trials in healthy volunteers and sickle cell patients, and faced delays with no published clinical results.^{26–28} Polymerized Hbs (PolyHbs), such as PolyHeme (Northfield Laboratories, Inc., Evanston, IL) and Hemopure (HbO₂ Therapeutics, Souderton, PA), were produced via glutaraldehyde cross-linking, generating large polymers (M_w 130–500 kDa, P_{50} = 28–38 mmHg). PolyHeme and Hemopure advanced to phase III clinical trials, with Hemopure receiving approval in South Africa for treating acute anemia in adults, though it did not gain approval by FDA in the United States.^{29–33} Hemolink, (Hemosol, Toronto, ON, Canada) a raffinose-cross-linked Hb (M_w 32 $\rightarrow$ 500 kDa, P_{50} $\sim$ 34 mmHg), was discontinued after evaluation in coronary artery bypass graft surgery.^{34–36}

Early polymerized human Hbs (PolyhHbs) faced significant challenges due to the presence of cell-free Hb and low-molecular-weight (LMW) polymeric species. To address these issues, our laboratory developed PolyhHbs synthesized in distinct tense (T) and relaxed (R) quaternary states through glutaraldehyde polymerization, followed by tangential flow filtration (TFF) to effectively remove cell-free Hb and LMW components.^{37–42} Our group was the first to demonstrate that by simply mixing T- and R-state PolyhHb molecules the oxygen affinity of the mixture can be tuned between that of pure T-state PolyhHb and R-state PolyhHb.⁴³ Subsequent studies confirmed that T-state PolyhHbs not only improved tissue oxygenation and maintained hemodynamic stability but also exhibited lower auto-oxidation rates and reduced toxicity relative to earlier HBOCs.^{39,44,45} Pilot-scale synthesis has also yielded high molecular weight (HMW) PolyhHb, supporting its potential for diverse biomedical applications.⁴⁶ These developments support the viability of PolyhHb as an effective and scalable therapeutic option for treating anemia, hemorrhagic shock, and improving overall tissue oxygenation.^{44,45,47,48}

A copolymer consisting of Hb and human serum albumin (HSA) was previously synthesized using a periodate-oxidized sugar derivative (oxidized ATP [oATP]), which reduced oxidative instability.⁴⁹ The Hb-HSA clusters can also serve as HBOCs by combining a Hb core with a HSA shell, improving both oxygen affinity and antioxidant properties.⁵⁰ Core-shell structures of Hb A (HbA) and HSA have been synthesized with varied oxygen affinities (P_{50} values of 8–35 mmHg).⁵¹ Specific clusters such as Hb-HSA3 and Hb-HSA4 enhance oxygen-binding properties and prolong circulation time in the bloodstream.⁵² HemoAct, a cluster of one Hb molecule bound to three HSA molecules, exhibits neuroprotective and antioxidant effects, emphasizing its therapeutic potential.⁵³ Hb-albumin clusters such as HbBv-HSA3 and HbA-HSA3 have shown efficacy in hemorrhagic shock resuscitation in rats, matching the outcomes of autologous blood transfusion.⁵⁴ Furthermore, core-shell Hb nanoparticles (HbNPs), with polymerized Hb cores coated by HSA, have demonstrated superior oxygen affinity and blood compatibility compared to RBCs.⁵⁵ Similarly, SFHbNPs, featuring a polymerized Hb core with an albumin shell, have proven effective in oxygen delivery and hemorrhagic shock therapy, with reduced heme-iron oxidation and good compatibility with human blood.⁵⁶

While the development of HMW PolyhHbs in our laboratory has significantly advanced HBOC technology, challenges remain in enhancing antioxidant capacity and in vivo circulation time.^{38,39,46} The integration of HSA with Hb, as demonstrated by Hb-HSA clusters and Hb-HSA nanoparticles, offers promising advantages, including improved oxygen affinity, enhanced antioxidant properties, and superior biocompatibility.^{50–56} Products such as HemoAct and core-shell Hb nanoparticles (HbNPs) have exhibited prolonged circulation times and reduced oxidative damage, suggesting that copolymerization of hHb with HSA could combine the benefits of high oxygen-carrying capacity with better antioxidant stability and reduced side effects. Therefore, the development of copolymerized Hb-HSA (Poly(hHb:HSA)) represents a logical and necessary progression to create safer, more effective HBOCs for clinical applications. In support of this goal, this study provides an extensive biophysical characterization comparing the precursor hHb to T-state Poly(hHb:HSA) conjugates.

2. MATERIALS AND METHODS

2.1. Materials. Glutaraldehyde (C₅H₈O₂) (25 wt %), sodium cyanoborohydride (NaCNBH₃) and sodium dithionite (Na₂S₂O₄) were sourced from Sigma-Aldrich (St. Louis, MO). Sodium phosphate monobasic (NaH₂PO₄), sodium phosphate dibasic (Na₂HPO₄), sodium chloride (NaCl), potassium chloride (KCl) and sodium hydroxide (NaOH) were obtained from Fisher Scientific (Pittsburgh, PA). Potassium ferricyanide (K₃Fe(CN)₆), potassium cyanide (KCN), and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). The Liqui-Cel MM Series G 543 Membrane Contactor was sourced from 3 M (St. Paul, MN). Modified poly(ether sulfone) (mPES) hollow fiber filter modules with pore sizes of 0.2 μ m (S02-P20U-05-N) and polysulfone (PS) hollow fiber filter modules with a pore size of 500 kDa (S02-S500-05-N), were supplied by Repligen (Rancho Dominguez, CA). HSA (AlbuRx 2S, CSL Behring, King of Prussia, PA) was purchased from The Ohio State Outpatient Pharmacy (Columbus, Ohio). Recently expired human RBC units were acquired from Impact Life (Davenport, IA).

2.2. Hb Purification. Human Hb (hHb) was purified via tangential flow filtration (TFF) as described previously in the literature.⁵⁷ The study involved the use of human-derived materials and was conducted with approval from The Ohio State University

Institutional Review Board (protocol #2022R00000046, A.F.P.). A total of 20 units of human red blood cells (RBCs) were combined and diluted with 0.9 wt % saline to achieve a hematocrit of $20\% \pm 2\%$. The suspension was subjected to six diafiltration cycles using a $0.65\ \mu\text{m}$ mPES hollow fiber filter with approximately 120 L of saline for thorough washing. The clarified RBC suspension was then concentrated to 8 L and lysed by adding phosphate buffer (3.75 mM, pH 7.4) at $4\ ^\circ\text{C}$, followed by stirring for 1 h. Cellular debris and large aggregates were removed by filtration through a 500 kDa hollow fiber module. The clarified hemolysate, consisting mainly of hHb ($\sim 98\%$) with minor amounts of endogenous RBC enzymes as previously described,⁵⁸ was further processed with a 50 kDa hollow fiber filter to achieve a hHb concentration of 200–250 mg/mL. The final hHb preparation was stored at $-80\ ^\circ\text{C}$ until needed.

2.3. Polymerization of Proteins. Polymerization of hHb was adapted from established protocols.^{40,59,60} Briefly, 30 g of purified hHb was diluted to 20 mg/mL in 150 mM phosphate-buffered saline (PBS, pH 7.4) in a 2 L vessel at $37\ ^\circ\text{C}$. Continuous nitrogen flow through a gas–liquid contactor and nitrogen-purged headspace enabled deoxygenation of hHb. The pO_2 was monitored using a RAPIDLab 248 blood gas analyzer (Siemens, Munich, Germany). After 30 min of deoxygenation, 300 mg sodium dithionite in 50 mL degassed PBS was added to the hHb. Upon confirmation of 0.0 mmHg pO_2 , glutaraldehyde (GA) was added at a rate of 2 mL/min to maintain a GA/protein molar ratio of 30:1. For Poly(hHb:HSA) conjugates (75% hHb: 25% HSA, 50% hHb: 50% HSA, and 25% hHb: 75% HSA mass ratios), the same polymerization protocol was used. For PolyHSA synthesis, 30 g HSA in PBS was polymerized at 20 mg/mL using GA at a 30:1 GA/HSA molar ratio. All reactions were conducted for 2 h at $37\ ^\circ\text{C}$ and quenched with sodium cyanoborohydride (7:1 molar ratio to GA), then stored at $4\ ^\circ\text{C}$ overnight.

2.4. Purification of Polymerized Protein. Polymerized protein purification involved two-stage tangential flow filtration (TFF), adapted from established protocols in the literature.^{40,59,60} Briefly, HMW materials were removed with a $0.2\ \mu\text{m}$ poly(ether sulfone) (PES) TFF module. The second TFF stage used a 500 kDa polysulfone (PS) membrane for diafiltration with PBS to remove unreacted proteins and LMW byproducts. Diafiltration continued for eight cycles or until the permeate protein concentration was $<0.5\ \text{mg/mL}$. The protein was then concentrated to $\sim 10\ \text{g/dL}$ and stored at $-80\ ^\circ\text{C}$.

2.5. Protein Quantification and Cyanomethemoglobin Assay. Protein concentration was measured using the Bradford assay by following the manufacturer's protocol (Coomassie Plus Protein Assay Kit, Pierce Biotechnology, Rockford, IL). Methemoglobin (metHb) levels were determined via the cyanomethemoglobin method,^{61,62} using an HP 8452A diode array spectrophotometer (Olivis Athens, GA).

2.6. Polymer Molecular Weight Estimation, Size, and Zeta Potential. Polymer estimated molecular weights were measured via size exclusion chromatography (SEC), employing a high-performance liquid chromatography (HPLC) system, as described in the literature.⁴⁶ Protein separation was conducted using an Ultimate 3000 system equipped with an Acclaim SEC-1000 ($4.6 \times 300\ \text{mm}$) column (Thermo Fisher Scientific, Waltham, MA). The absorbance at 413 nm was used for monitoring PolyhHb and Poly(hHb:HSA) conjugates; while the absorbance at 280 nm was used for monitoring PolyHSA. The hydrodynamic diameter was measured by dynamic light scattering (DLS) with a Malvern Zetasizer Pro (Malvern Panalytical Inc., Westborough, MA). Protein samples were diluted in deionized water, and light scattering was measured at 637 nm with a 90° detection angle. The zeta potential of the proteins was analyzed using a Malvern Analytical Zetasizer Helix (Worcestershire, UK) by diluting samples in deionized water to $\sim 1\ \text{mg/mL}$.

2.7. Oxygen Equilibrium Curve. Oxygen equilibrium curves (OECs) were measured using a Hemox Analyzer (TCS Scientific Corp., New Hope, PA) at $37\ ^\circ\text{C}$ and pH 7.4 in accordance with established methodologies.⁴⁶ Samples were diluted to 2 mg/mL in Hemox buffer with 20 μL each of Additive A and antifoaming agent.

The Hill equation was employed to model the OEC data, from which oxygen affinity (P_{50}) and the cooperativity coefficient (n) were regressed.

2.8. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein samples (0.25 mg/mL) were mixed 1:1 with Tris-glycine SDS buffer (Fisher Scientific, Pittsburgh, PA), with or without DTT (0.1 M), and heated at $85\ ^\circ\text{C}$ for 10–15 min. Twenty μL was loaded onto a 10–20% gradient precast tris-glycine gel and electrophoresed at 220 V for 40 min. Gels were stained with Coomassie blue (Bio-Rad Laboratories) and destained using a solution of 60% DI water, 30% methanol, 10% acetic acid. Gel images were captured, and protein bands were compared to a molecular weight standard ladder.

2.9. Auto-Oxidation Kinetics. The auto-oxidation kinetics was assessed using UV visible spectrophotometry, following a previously reported protocol.⁴⁶ Briefly, samples at 12.5 mg/mL (0.77 mM, heme basis) were incubated in 150 mM PBS, pH 7.4 at $37\ ^\circ\text{C}$ using a quartz cuvette with a 0.1 cm path length. The absorbance at 630 nm, indicating metHb, was recorded every 30 min for 24 h. Kinetic analysis was performed using a custom R script, applying a first-order kinetic model to determine the auto-oxidation rate constant (k_{ox}), expressed in units of h^{-1} .

2.10. Heme Loss Kinetics. The kinetics of heme dissociation was evaluated via UV–visible spectrophotometry, following established protocols described in the literature.⁴⁶ Briefly, samples were oxidized using an excess of potassium ferricyanide and desalted using Sephadex G-25 (Cytiva, Marlborough, MA) to yield ferric Hb. Oxidized samples were then diluted to 2 μM ferric hHb (Hb tetramer basis) in 200 mM potassium phosphate buffer, pH 7.4 with 600 mM sucrose. Human hemopexin (Hpx) (5 $\times$ molar excess) was added, and spectra were recorded every 2 min for 15 h at $37\ ^\circ\text{C}$ using a quartz cuvette with a 1.0 cm path length. The absorbance change at 414 nm was fit to a double exponential decay to determine heme dissociation rate constants, expressed in units of h^{-1} .

2.11. Stopped-Flow Spectroscopy. The kinetics of oxygen dissociation and haptoglobin (Hp) binding were investigated using an SX-20 microvolume stopped-flow spectrophotometer (Applied Photophysics, Charlotte, NC), following established methodologies reported in the literature.⁴⁶

2.11.1. Oxygen Offloading Kinetics. Oxygen offloading kinetics were determined by rapidly mixing oxygenated solutions (30 μM , Hb tetramer basis) with a 1.5 mg/mL solution of sodium dithionite prepared in deoxygenated 150 mM PBS, pH 7.4. The absorbance at 437.5 nm was recorded, averaged from ten traces, and fitted to a first-order exponential decay model using the Applied Photophysics software to determine the oxygen dissociation rate constant ($k_{\text{O}_2\text{off}}$), expressed in units of s^{-1} .

2.11.2. Haptoglobin Binding Kinetics. The binding kinetics of Hp to hHb/Poly(hHb:HSA) was characterized by measuring changes in intrinsic fluorescence emission at 310 nm and with an excitation wavelength at 285 nm, indicative of Hp–Hb complex formation.⁶³ Hp (0.25 μM) was mixed with varying concentrations of hHb/Poly(hHb:HSA) (5, 10, 15, and 20 μM , Hb tetramer basis). Each condition was ten traces were collected, and the fluorescence traces were averaged prior to analysis. The averaged kinetic profiles were fitted to a single-exponential model to obtain the pseudo-first-order rate constants. These values were plotted as a function of Hb/Poly(hHb:HSA) concentration, and the slope of the linear fit was used to calculate the second-order association rate constant for Hp binding ($k_{\text{Hp-Hb}}$), reported in units of $\mu\text{M}^{-1}\ \text{s}^{-1}$.

2.12. Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were acquired using a JASCO J-815 spectropolarimeter (JASCO, Easton, MD). Far-UV spectra (190–260 nm) for secondary structure were recorded at 0.5 mg/mL ($\sim 30\ \mu\text{M}$ heme basis) of protein in 150 mM PBS, pH 7.4, in a 0.1 cm path length quartz cuvette. The near-UV spectra (250–500 nm), used to evaluate tertiary structure, was collected from protein samples adjusted to 1.5 mg/mL ($\sim 90\ \mu\text{M}$ heme basis) under identical buffer conditions and cell path length. Each sample was scanned three times, and results

Table 1. Biophysical and Biochemical Parameters of hHb, HSA, T-State PolyhHb, T-State Poly(hHb:HSA) Conjugates, and PolyHSA

parameter	precursor hHb (<i>n</i> = 3)	polyhHb ₁₀₀ (<i>n</i> = 3)	poly(hHb ₇₅ :HSA ₂₅) (<i>n</i> = 3)	poly(hHb ₅₀ :HSA ₅₀) (<i>n</i> = 3)	poly(hHb ₂₅ :HSA ₇₅) (<i>n</i> = 3)	precursor HSA (<i>n</i> = 3)	polyHSA ₁₀₀ (<i>n</i> = 3)
mass/batch (g) ^a	N/A	13.0 ± 0.6	11.8 ± 0.2	7.7 ± 1.3	5.2 ± 0.3	N/A	0.5 ± 0.0
yield (%) ^b	N/A	43 ± 2	39 ± 1	26 ± 4	17 ± 1	N/A	2 ± 0
MetHb (%)	N/A	2.43 ± 0.72	2.27 ± 0.55	2.40 ± 0.26	2.83 ± 0.64	N/A	N/A
<i>P</i> ₅₀ (mm Hg)	0.50 ± 0.17	45.99 ± 2.77	49.04 ± 0.95	41.79 ± 0.81	35.92 ± 3.89	N/A	N/A
cooperativity (<i>n</i> ₅₀)	2.86 ± 0.09	0.98 ± 0.05	0.99 ± 0.01	1.03 ± 0.03	1.02 ± 0.02	N/A	N/A
<i>k</i> _{O₂off} (s ^{−1})	37.3 ± 0.3	54.8 ± 5.2	63.1 ± 0.5	59.0 ± 1.4	55.8 ± 2.6	N/A	N/A
<i>k</i> _{Hb-Hb} (μM ^{−1} s ^{−1})	0.127 ± 0.005	0.007 ± 0.001	0.005 ± 0.000	0.004 ± 0.000	0.004 ± 0.000	N/A	N/A
<i>D</i> _{eff} (nm)	5.9 ± 0.1	44.0 ± 5.2	29.6 ± 2.3	21.5 ± 1.9	14.5 ± 1.1	6.9 ± 0.3	24.6 ± 5.8
average <i>M</i> _w (kDa)	64.5	2034 ± 169	2024 ± 262	875 ± 84	466 ± 56	92.1 ± 0.7	peak 1 3270 ± 14 peak 2 486 ± 42
<i>k</i> _{heme release} (h ^{−1})							
<i>k</i> _{fast}	5.08 ± 0.32	0.63 ± 0.04	0.56 ± 0.03	0.41 ± 0.03	0.18 ± 0.01	N/A	N/A
<i>k</i> _{slow}	0.48 ± 0.001	0.03 ± 0.005	0.08 ± 0.001	0.03 ± 0.01	0.04 ± 0.001	N/A	N/A
<i>k</i> _{ox} (h ^{−1})							
<i>k</i> _{fast}	0.010 ± 0.001	0.014 ± 0.001	0.015 ± 0.002	0.014 ± 0.001	0.012 ± 0.001	N/A	N/A
<i>k</i> _{slow}	N/A	0.009 ± 0.001	0.009 ± 0.001	0.008 ± 0.001	0.008 ± 0.002	N/A	N/A
catalase activity (Units/mL)	6.67 ± 2.40	3.13 ± 0.42	2.20 ± 0.21	2.02 ± 0.78	2.66 ± 0.38	N/A	N/A
catalase activity (Units/mg)	0.0290 ± 0.0105	0.0326 ± 0.0046	0.0247 ± 0.0031	0.0210 ± 0.0083	0.0324 ± 0.0047	N/A	N/A
thermal stability (<i>T</i> _m) (°C)	66.2 ± 0.4	73.5 ± 0.2	69.5 ± 2.4	70.9 ± 2.6	71.9 ± 0.8	70.3 ± 0.3	77.8 ± 0.5
zeta potential (mV)	−0.6 ± 0.4	−18.8 ± 1.8	−23.2 ± 1.3	−27.0 ± 1.7	−26.7 ± 1.9	−15.0 ± 1.7	−26.1 ± 4.1

^aMass/batch refers to the total mass of protein recovered after formulation. ^bYield (%) refers to the total mass of protein recovered after purification based on the starting mass of protein.

were averaged. Spectra were corrected by subtracting buffer controls. CD spectra were reported in molar ellipticity units ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$).

2.13. Thermal Stability. Thermal unfolding was monitored via CD spectroscopy at 222 nm (which corresponds to the α -helix content) from 25 to 90 °C. Protein samples were diluted to 0.5 mg/mL in 150 mM PBS, pH 7.4 in a quartz cuvette with a 0.1 cm path length, were heated at 2.5 °C/min with 3 min equilibration at each step prior to data acquisition. Baseline subtraction was performed using 150 mM PBS at pH 7.4.

2.14. MALDI-TOF Spectrometry. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was used to analyze protein mass by following previously established protocols.⁴⁶ Protein samples (0.25 mg/mL in PBS, pH 7.4) were mixed 1:5 with a α -cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg/mL in 50% acetonitrile, 0.1% TFA). A volume of 2 μL of the protein mixture were spotted onto a target plate, air-dried. Samples were analyzed using a Bruker Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Spectral data were processed and interpreted using Bruker FlexAnalysis software.

2.15. Catalase Activity Assay. The catalase (CAT) activity of Poly(hHb:HSA) was evaluated via UV–visible spectrophotometry following established protocols.^{46,64} A standard curve (0, 1, 2, 3, 4, 5, and 10 U/mL) was generated by measuring the decrease in absorbance at 240 nm after incubation with 15 mM H_2O_2 . Protein samples (1 μM , heme basis) were mixed with 15 mM H_2O_2 in 150 mM PBS (pH 7.4) using a quartz cuvette with a 0.1 cm path length and monitored at 240 nm for 3 min. CAT activity was quantified using the standard curve. All measurements were performed in triplicate.

2.16. RBC Aggregation, Viscosity and Colloid Osmotic Pressure Measurement. RBC aggregation was evaluated using a Myrenne aggregometer (Myrenne GmbH, Roetgen, Germany). male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing between 250 and 400 g were used as blood donors. Animals were handled according to the NIH Guide for Care and Use of Laboratory Animal blood collection was approved by UCSD's Institution for Animal Care and Use Committee (S00147). Briefly, animals were anesthetized with isoflurane (5% induction and 2.5% maintenance, Drägerwerk AG, Lübeck, Germany), and a left femoral arterial catheter was placed to collect 30% of the animal's blood volume, estimated as 7% of body mass over a period of 10 min into heparinized syringes. After blood collection, animals were humanely euthanized via sodium pentobarbital (300 mg/kg) followed by bilateral thoracotomy. Measurements were performed at low shear rates using the M1 index on 2:1 (v/v) mixtures of rat whole blood and Poly(hHb:HSA) copolymer solutions. Each formulation was tested with five independent samples, three readings per sample, and repeated using blood from four different donors to ensure reproducibility. The viscosity dependence on concentration was determined using steady-shear rheometry (Discovery HR-2, TA Instruments) at 37 °C with a 2° cone-and-plate geometry ($R = 20$ mm, truncation ≈ 50 μm). Poly(hHb:HSA) samples at 50 $\text{mg}\cdot\text{mL}^{-1}$ and 100 $\text{mg}\cdot\text{mL}^{-1}$ in lactated Ringer's buffer were subjected to flow sweeps from 1000 $\rightarrow$ 10 $\rightarrow$ 1000 s^{-1} under normal-force control. For each shear rate, viscosity values were averaged between ascending and descending sweeps once steady state was reached. The concentration-scaling exponent $\alpha(\dot{\gamma})$ was computed as $\ln(\eta_{100}/\eta_{50})/\ln 2$, and its dependence on $\ln \dot{\gamma}$ was modeled by linear regression to extract the slope (s) and intercept (a). These parameters were related to flow-behavior indices and consistency ratios between concentrations. Data showing slip or instrument artifacts were excluded. Solution colloidal oncotic pressure (COP) was measured using a 4420 Colloid Osmometer (Wescor, Logan, UT) equipped with a 30 kDa molecular cutoff membrane.

2.17. Statistical Analysis. Data are presented as mean values $\pm$ standard deviation, where appropriate. Statistical comparisons were conducted using Student's t -test, one-way analysis of variance (ANOVA), and Tukey's post hoc test as applicable. All analyses were performed using JMP Pro (Version 16, SAS Institute Inc., Cary, NC), with a threshold for statistical significance set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Production Yield. The mass/batch (g) and production yields (%) of T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) conjugates and PolyHSA₁₀₀ are reported in Table 1. Among these, T-state PolyhHb₁₀₀ demonstrated the highest average yield at $43\% \pm 2$, which was significantly higher than the yields of all other formulations ($p < 0.0001$). Notably, this yield is consistent with previously reported values for benchtop scale 30:1 T-state PolyhHb batches produced in our laboratory.³⁸ The yield decreased progressively with increasing HSA mass fraction. T-state Poly(hHb₇₅:HSA₂₅) followed closely with an average yield of $39\% \pm 1$, showing no statistically significant difference compared to T-state PolyhHb₁₀₀ ($p = 0.3770$). However, the yield dropped more sharply for T-state Poly(hHb₅₀:HSA₅₀), averaging $26\% \pm 4$, which was significantly lower than T-state PolyhHb₁₀₀ ($p < 0.0001$). The trend continued with T-state Poly(hHb₂₅:HSA₇₅), yielding only $17\% \pm 1$, also significantly lower than T-state PolyhHb₁₀₀ ($p < 0.0001$). The lowest production was observed for PolyHSA₁₀₀, with an average yield of just 2%, representing a highly significant reduction compared to all formulations ($p < 0.0001$).

The findings indicate that higher HSA content (i.e., mass fraction) is associated with a substantial reduction in polymer yield, with the lowest yield observed in the formulation containing only HSA. This trend may be attributed to the cross-linking conditions used; specifically, a 30:1 glutaraldehyde to hHb molar ratio appears to be efficient for hHb polymerization. In contrast, earlier studies from our laboratory reported higher yields of PolyHSA only at higher cross-link densities, 43:1 and $>$ glutaraldehyde to HSA ratios, which is more suitable for effective HSA cross-linking.⁶⁰

In addition, the feed mass composition of hHb:HSA does not directly translate to the same mass composition in the final polymerized product because the overall polymerization yield is not 100%. To evaluate this, the relative proportion of hHb in the purified copolymer was determined as the ratio of hHb concentration (from the cyanomethemoglobin assay) to total protein concentration (from the Bradford assay). The calculated hHb mass fractions were approximately ~ 1.0 for Poly(hHb₁₀₀), ~ 0.78 for Poly(hHb₇₅:HSA₂₅), ~ 0.60 for Poly(hHb₅₀:HSA₅₀), and ~ 0.38 for Poly(hHb₂₅:HSA₇₅). These results demonstrate that the hHb mass content within the recovered copolymer decreases progressively with increasing HSA mass fraction, consistent with reduced incorporation efficiency of HSA under the same cross-linking conditions. This deviation arises because losses occur during the polymerization and purification steps, including incomplete cross-linking, removal of unreacted or low-molecular-weight copolymer during TFF, and potential minor aggregation or precipitation. Consequently, the Hb:HSA ratio in the final copolymer reflects the combined effects of yield and protein-specific reaction efficiency rather than the initial feed composition.

3.2. Methemoglobin Level. The results of metHb formation is presented in Table 1. The precursor hHb exhibited the lowest metHb formation ($0.50\% \pm 0.17$). This is likely due to its purification from near-expired human RBCs and subsequent storage at -80 °C, which preserved its functional integrity. These findings are consistent with earlier reports showing that native bovine Hb typically maintains low metHb levels ($< 1\%$).⁶⁵ In comparison, T-state PolyhHb₁₀₀ and

various T-state Poly(hHb:HSA) conjugates exhibited elevated metHb levels relative to the precursor hHb, aligning with prior studies that reported increased metHb in T-state PolyhHb formulations.^{37,38,59} Specifically, T-state PolyhHb₁₀₀ exhibited the highest metHb level ($2.43\% \pm 0.72$), with T-state Poly(hHb₂₅:HSA₇₅) following closely ($2.83\% \pm 0.64$). The formulations T-state Poly(hHb₇₅:HSA₂₅) and T-state Poly(hHb₅₀:HSA₅₀) yielded intermediate metHb values of $2.27\% \pm 0.55$ and $2.40\% \pm 0.26$, respectively. The statistical analysis of metHb formation indicates that T-state PolyhHb₁₀₀ and all T-state Poly(hHb:HSA) conjugates show a statistically significant increase in metHb formation compared to the precursor hHb, with *p*-values ranging from 0.0018 to 0.0124 (all *p* < 0.05). This suggests that polymerization of hHb, regardless of the ratio with HSA, significantly alters its oxidation profile. However, there are no statistically significant differences in metHb formation among the various T-state Poly(hHb:HSA) conjugates formulations themselves (all *p* > 0.05), indicating that the mass ratio of hHb to HSA in the polymer complexes does not significantly impact metHb levels.

The metHb levels observed in T-state PolyhHb₁₀₀ and the various Poly(hHb:HSA) conjugates in this study remained below 3–4%, which is much less than the commonly referenced upper limit of 10% metHb for HBOCs.⁶⁶ This range also compares favorably to commercial HBOCs such as PolyHeme, which typically exhibits metHb levels under <8%.¹¹ As such, the metHb levels in these formulations meet established oxygen carrying capacity criteria for HBOC development. To minimize metHb formation, several precautionary measures were implemented. First, the purified precursor hHb starting material had a metHb content below 1%, ensuring a low-oxidation baseline. Second, the TFF process used for fractionating the PolyhHb mixtures was performed in a cold environment (4 °C), which helped limit heme oxidation throughout processing.

3.3. Oxygen Equilibrium Curves. The OECs of precursor the hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates was characterized using a Hemox Analyzer. Representative OEC traces are presented in Figure 1, with corresponding oxygen affinity (*P*₅₀) and cooperativity

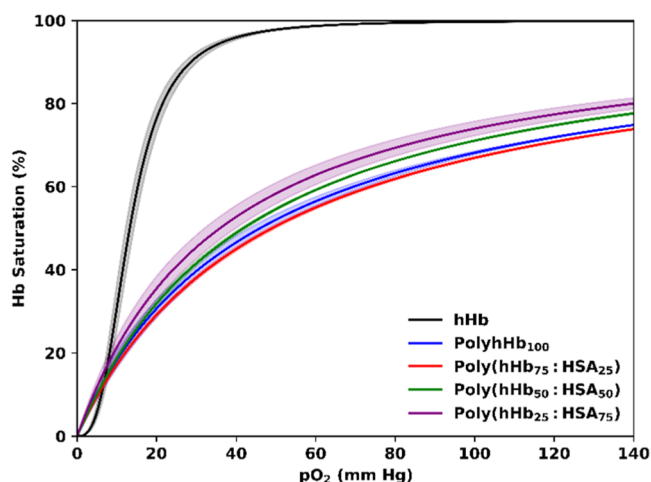


Figure 1. OECs for the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates were obtained using a Hemox analyzer in Hemox buffer at pH 7.4 and 37 °C. Curves represent average values, with standard deviations indicated by shaded regions corresponding to each curve's color.

coefficients (*n*₅₀) summarized in Table 1. The precursor hHb displayed a characteristic sigmoidal OEC with a low *P*₅₀ (13.26 ± 1.07 mmHg), indicative of high oxygen affinity, consistent with previously reported findings.^{37,43,46} In contrast, T-state PolyhHb₁₀₀ exhibited a significantly elevated *P*₅₀ (45.99 ± 2.77 mmHg), reflecting a substantial decrease in oxygen affinity relative to the precursor hHb (*p* < 0.0001), in agreement with earlier studies.^{37,46,65} This trend continued with T-state Poly(hHb₇₅:HSA₂₅), which exhibited the highest *P*₅₀ value (49.04 ± 0.95 mmHg), significantly greater than that of the precursor hHb (*p* < 0.0001). Additionally, T-state Poly(hHb₇₅:HSA₂₅) demonstrated a significantly higher *P*₅₀ compared to T-state Poly(hHb₅₀:HSA₅₀) (41.79 ± 0.81 mmHg, *p* = 0.0185) and T-state Poly(hHb₂₅:HSA₇₅) (35.92 ± 3.89 mmHg, *p* = 0.0002). However, the difference in *P*₅₀ between T-state Poly(hHb₇₅:HSA₂₅) and T-state Poly(hHb-100) was not statistically significant (*p* = 0.4975).

The cooperativity of the precursor hHb was 2.86 ± 0.09 , indicative of high cooperativity between the Hb subunits for oxygen binding, and consistent with previously reported literature.^{43,46} In contrast, T-state PolyhHb₁₀₀ displayed a significantly reduced cooperativity (0.98 ± 0.05 ; *p* < 0.0001 vs the precursor hHb), suggesting a substantial loss of allosteric behavior due to polymerization, and consistent with previously reported literature.^{43,46} Progressive substitution of HSA in the polymer mixture led to only minor variations in cooperativity. T-state Poly(hHb₇₅:HSA₂₅), Poly(hHb₅₀:HSA₅₀), and Poly(hHb₂₅:HSA₇₅) exhibited *n*₅₀ values of 0.99 ± 0.01 , 1.03 ± 0.03 , and 1.02 ± 0.02 , respectively, with no statistically significant differences among them (*p* > 0.05 for all comparisons between the HSA-containing groups). However, all these formulations remained significantly less cooperative than the precursor hHb (*p* < 0.0001 for each).

In this study, T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates demonstrated elevated *P*₅₀ values, reflecting reduced oxygen affinity compared to the precursor hHb. This shift is attributed to the complete deoxygenation of hHb prior to, during, and following polymerization which results conformationally stabilized the low-affinity T-state quaternary structure of hHb.^{37,67} As a consequence, these PolyhHbs exhibit significantly higher *P*₅₀ values relative to unmodified hHb. The cooperativity (*n*₅₀) values of T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates were consistently below 1.0, markedly lower than that of the precursor hHb. This reduction is attributed to extensive intra- and intermolecular cross-linking via glutaraldehyde, which immobilizes the Hb quaternary structure, thereby limiting conformational flexibility and impairing cooperative oxygen binding.^{67,68}

Among the formulations tested, T-state PolyhHb₁₀₀ showed a *P*₅₀ of 45.99 ± 2.77 mmHg, which is similar to O-R-polyHbA₀ (50.9 mmHg)⁶⁹ and exceeds the oxygen affinity of both T-state polymerized bovine Hb (PolyhHb, 41 mmHg)⁶⁵ and Hemopure (38 mmHg).¹¹ Notably, the T-state Poly(hHb₇₅:HSA₂₅) formulation exhibited the highest *P*₅₀ (49.04 ± 0.95 mmHg), suggesting an even lower oxygen affinity. In contrast, formulations containing higher proportions of HSA, T-state Poly(hHb₅₀:HSA₅₀) and T-state Poly(hHb₂₅:HSA₇₅) displayed reduced *P*₅₀ values of 41.79 ± 0.81 mmHg and 35.92 ± 3.89 mmHg, respectively. These values approach those of commercial HBOCs such as HBOC-201 (38 mmHg)⁷⁰ and Oxyglobin (35.1 mmHg),⁷¹ indicating partial restoration of oxygen-binding affinity. These findings are of clinical relevance,

as the oxygen-release characteristics of T-state PolyhHb formulations fall within the therapeutic window required for effective tissue oxygenation, particularly in applications such as hemorrhagic shock resuscitation and other conditions characterized by extensive hypoxia.^{67,72,73}

3.4. Oxygen Offloading Kinetics. The oxygen offloading kinetics ($k_{O_2, \text{off}}$) of the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates were evaluated using stopped-flow UV–visible spectroscopy. The representative kinetic traces depicted in Figure 2, along with the corresponding rate constants summarized in Table 1.

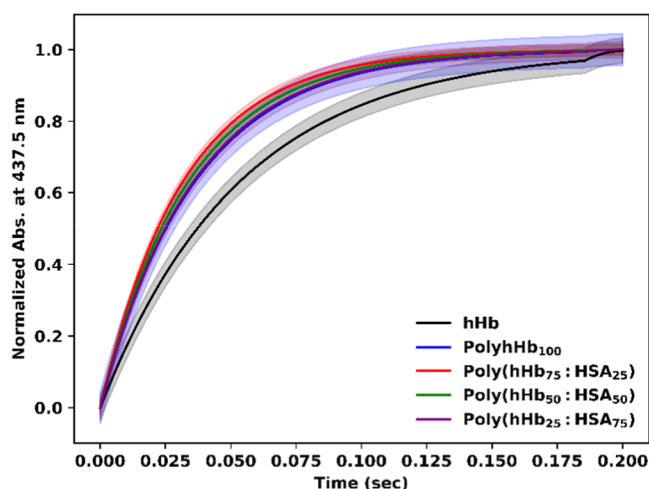


Figure 2. Oxygen dissociation kinetics for the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates were measured using stopped-flow spectroscopy by rapidly mixing the solutions with 1.5 mg/mL of sodium dithionite for deoxygenation. The absorbance at 437.5 nm was monitored in 150 mM PBS (pH 7.4) at room temperature. The data represents the average of 10 kinetic traces, with standard deviations indicated by shaded regions corresponding to each curve's color.

The precursor hHb exhibited the slowest oxygen dissociation rate constant, with an $k_{O_2, \text{off}}$ of $37.3 \pm 0.3 \text{ s}^{-1}$, these observations are consistent with the literature.⁴³ Upon polymerization to T-state PolyhHb₁₀₀, the oxygen offloading rate increased significantly to $54.8 \pm 5.2 \text{ s}^{-1}$ ($p < 0.0001$ versus hHb), indicating enhanced oxygen release kinetics. This finding is consistent with previous reports and reflects the inherently lower oxygen affinity of T-state PolyhHb, which promotes accelerated O_2 dissociation.^{37,65} Further, T-state Poly(hHb₇₅:HSA₂₅) achieved the highest oxygen offloading rate constant of $63.1 \pm 0.5 \text{ s}^{-1}$, significantly exceeding both the precursor hHb ($p < 0.0001$) and T-state PolyhHb₁₀₀ ($p = 0.0249$). However, increasing the proportion of HSA beyond 25% led to a moderate decline in $k_{O_2, \text{off}}$, with T-state Poly(hHb₅₀:HSA₅₀) and T-state Poly(hHb₂₅:HSA₇₅) exhibiting values of $59.0 \pm 1.4 \text{ s}^{-1}$ and $55.8 \pm 2.6 \text{ s}^{-1}$, respectively. Despite this decline, both constructs offloaded O_2 significantly faster than the precursor hHb ($p < 0.0001$). Importantly, the reduction in the O_2 offloading rate constant from Poly(hHb₇₅:HSA₂₅) to the higher mass fraction Poly(hHb:HSA) formulations was not statistically significant ($p = 0.4041$ vs Poly(hHb₅₀:HSA₅₀); $p = 0.0514$ vs Poly(hHb₂₅:HSA₇₅). Additionally, Poly(hHb₂₅:HSA₇₅) was not significantly different from PolyhHb₁₀₀ ($p = 0.9887$), underscoring the limited

effect of higher HSA mass fraction in the hHb:HSA copolymer. These findings suggest that a 25% mass fraction HSA incorporation represents the optimal ratio for maximizing the oxygen release performance in these T-state Poly(hHb:HSA) conjugates. Overall, the data support the conclusion that HSA copolymerization modulates oxygen delivery kinetics, with the most favorable outcome observed in the T-state Poly(hHb₇₅:HSA₂₅) formulation.

In this study, T-state PolyhHb₁₀₀ construct exhibited a $k_{O_2, \text{off}}$ of $54.8 \pm 5.2 \text{ s}^{-1}$, which is comparable to that of T-state PolyhHb (53 s^{-1}) as reported in the literature,⁶⁵ and closely aligns with Hemopure ($52.2 \pm 0.2 \text{ s}^{-1}$).⁶⁴ Notably, the T-state Poly(hHb₇₅:HSA₂₅) formulation demonstrated an elevated $k_{O_2, \text{off}}$ of $63.1 \pm 0.5 \text{ s}^{-1}$, surpassing that of T-state PolyhHb₁₀₀ and closely approximating the value reported for oxyglobin (61.8 s^{-1}),⁷¹ suggesting comparable oxygen release kinetics. However, further increases in the HSA mass fraction beyond 25% resulted in a progressive decline in $k_{O_2, \text{off}}$ with values of $59.0 \pm 1.4 \text{ s}^{-1}$ and $55.8 \pm 2.6 \text{ s}^{-1}$ observed for the T-state Poly(hHb₅₀:HSA₅₀) and T-state Poly(hHb₂₅:HSA₇₅) constructs, respectively. Despite this reduction, these formulations continued to exhibit oxygen offloading rate constants greater than native hHb and remained within the performance range of other clinically relevant HBOCs such as Hemopure ($52.2 \pm 0.2 \text{ s}^{-1}$)⁶⁴ and PolyhHb (53 s^{-1}).⁶⁵ Importantly, none of the T-state PolyhHb₁₀₀ or Poly(hHb:HSA) variants reached the elevated oxygen offloading rate constants reported for high-oxygen dissociation commercial HBOCs such as PolyHeme ($84.0 \pm 0.3 \text{ s}^{-1}$)⁶⁴ or Hemolink (130 s^{-1}),⁶⁹ indicating that although polymerization and moderate HSA incorporation enhanced oxygen release kinetics, these modifications preserve a moderate oxygen affinity, which may be advantageous for maintaining controlled oxygen delivery in vivo.

3.5. Haptoglobin Binding Kinetics. The harmful impact of circulating cell-free hHb can be reduced through its interaction with the plasma protein Hp, which specifically binds to hHb and LMW PolyhHb, thereby aiding in their clearance from the bloodstream.⁷⁴ The kinetics of Hp binding to the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates was evaluated by monitoring the decrease in fluorescence following binding Hp. A pronounced change in fluorescence was detected when Hp bound to the precursor hHb, whereas smaller changes were observed with T-state PolyhHb₁₀₀ and Poly(hHb:HSA) conjugates as shown in Figure 3A. Figure 3B displays the pseudo-first-order binding rate constants of Hp with each of these hHb polymers and shows how the rate constants vary with increasing concentrations of hHb or T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates. Linear regression analysis of these data yielded second-order binding rate constants ($k_{\text{Hp-Hb}}$) for each formulation, as listed in Table 1, indicate a significant reduction in Hp-binding kinetics upon polymerization and further reduction with increasing mass fraction of HSA in the Poly(hHb:HSA) conjugates. The second-order rate constant for Hp binding to the precursor hHb was determined to be $0.127 \pm 0.005 \mu\text{M}^{-1}\text{s}^{-1}$, which aligns closely with previously reported values for hHb ($\sim 0.129 \mu\text{M}^{-1}\text{s}^{-1}$).^{63,64} This binding rate constant was significantly greater than those observed for T-state PolyhHb₁₀₀ and all Poly(hHb:HSA) conjugates ($p < 0.0001$). T-state PolyhHb₁₀₀ showed a reduction in Hp binding, with a rate constant of $0.007 \pm 0.001 \mu\text{M}^{-1}\text{s}^{-1}$ ($p < 0.0001$, compared to the precursor hHb). Further incorpo-

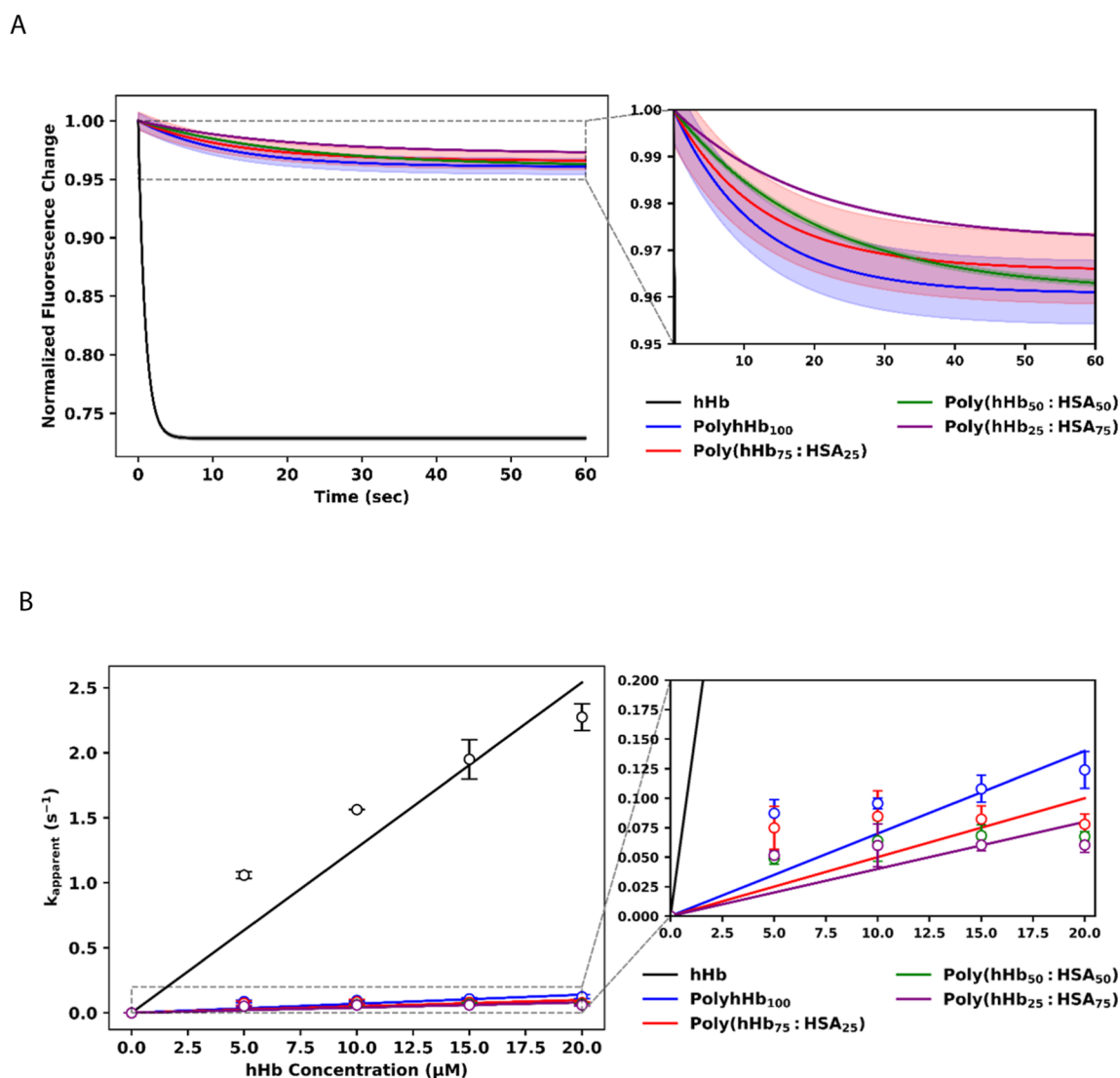


Figure 3. Stopped-flow measurements of Hp binding kinetics to precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates. (A) Representative fluorescence kinetic traces for the reaction of 0.25 μM Hp (based on hHb tetramer) with 5.0 μM of each PolyhHb/Poly(hHb:HSA), fitted to a monoexponential equation (solid lines). The data represent the average of 10 kinetic traces with standard deviations indicated by shaded regions corresponding to each curve's color. (B) Pseudo-first-order rate constants were plotted against hHb/PolyhHb/Poly(hHb:HSA) concentrations (Hb tetramer basis) to derive second-order rate constants for Hp-binding across all samples.

ration of HSA into the T-state PolyhHb structure progressively decreased the binding rate constants: $0.005 \pm 0.000 \mu\text{M}^{-1}\text{s}^{-1}$ for T-state Poly(hHb₇₅:HSA₂₅), $0.004 \pm 0.000 \mu\text{M}^{-1}\text{s}^{-1}$ for T-state Poly(hHb₅₀:HSA₅₀), and $0.004 \pm 0.000 \mu\text{M}^{-1}\text{s}^{-1}$ for T-state Poly(hHb₂₅:HSA₇₅). Statistical comparisons confirm that all polymerized forms had significantly reduced Hp binding relative to the precursor hHb (all comparisons with $p < 0.0001$). However, among the PolyhHb/Poly(hHb:HSA) groups, there were no statistically significant differences in binding kinetics. For instance, the comparisons between T-state PolyhHb₁₀₀ and T-state Poly(hHb₂₅:HSA₇₅), T-state Poly(hHb₅₀:HSA₅₀), and T-state Poly(hHb₇₅:HSA₂₅) yielded p -values of 0.3874, 0.3874, and 0.7552, respectively, indicating no significant differences. Similarly, comparisons among the T-state Poly(hHb:HSA) conjugates also showed no significant differences ($p = 0.9560$ to 1.0000). These results suggest that polymerization, regardless of HSA mass fraction, leads to a marked reduction in Hp binding compared to the precursor

hHb, but variations in HSA mass fraction among the polymers do not significantly influence this reduced Hp-binding affinity.

T-state PolyhHb₁₀₀ exhibited a reduced Hp binding rate compared to precursor hHb, likely due to intra- and intermolecular glutaraldehyde cross-links that limit the surface accessibility of Hb subunits for Hp binding. Previous studies have demonstrated that both the type of cross-linking and the hHb quaternary structure significantly influence Hp-binding affinity.⁶³ Additionally, extensive diafiltration using a 500 kDa membrane likely contributed to the reduced Hp-binding by effectively removing cell-free hHb and LMW hHb species, which are primary targets for Hp. For Poly(hHb:HSA) conjugates, Hp-binding rate constants further declined with increasing HSA mass fraction. This reduction may be attributed to two main factors: (1) steric hindrance, where HSA moieties physically obstruct Hp access to hHb binding sites, and (2) decreased hHb density per particle, as increasing the HSA mass fraction in the hHb:HSA copolymer result in fewer hHb molecules per conjugate. While the hHb

concentration was defined in μM across experiments, implying that the total number of available hHb-binding sites remained constant, the distribution of those sites changed. Specifically, increasing HSA content leads to a greater number of smaller hHb clusters with reduced hHb density, thereby limiting the effective accessibility of binding sites for Hp. Compared to the commercial HBOC (PolyHeme), which exhibits a Hp-binding rate constant of $0.003 \pm 0.000 \mu\text{M}^{-1} \text{s}^{-1}$,⁶⁴ the T-state PolyhHb₁₀₀ synthesized in the present study demonstrated a moderately elevated rate constant of $0.007 \pm 0.001 \mu\text{M}^{-1} \text{s}^{-1}$. Among the Poly(hHb:HSA) conjugates, the Hp-binding rate constants decreased with increasing HSA mass fraction, with values of $0.005 \pm 0.000 \mu\text{M}^{-1} \text{s}^{-1}$ for T-state Poly(hHb₇₅:HSA₂₅), $0.004 \pm 0.000 \mu\text{M}^{-1} \text{s}^{-1}$ for T-state Poly(hHb₅₀:HSA₅₀), and $0.004 \pm 0.000 \mu\text{M}^{-1} \text{s}^{-1}$ for T-state Poly(hHb₂₅:HSA₇₅). These values are comparable to that of PolyHeme, indicating similar Hp interaction kinetics. In contrast, the commercial HBOCs Hemopure and Oxyglobin exhibited significantly higher Hp-binding rate constants of $0.010 \pm 0.001 \mu\text{M}^{-1} \text{s}^{-1}$ and $0.011 \pm 0.001 \mu\text{M}^{-1} \text{s}^{-1}$, respectively,⁶⁴ suggesting more rapid Hp-binding kinetics relative to all formulations assessed in the current study.

3.6. Polymer Size and Estimated Molecular Weight.

The hydrodynamic diameter (D_{eff}) and estimated M_{W} of the precursor hHb, T-state PolyhHb₁₀₀, various T-state Poly(hHb:HSA) conjugates, HSA, and PolyHSA₁₀₀ in this study was measured using DLS and SEC-HPLC as shown in Figure 4 A and B, respectively and the corresponding D_{eff} and M_{W} are summarized in Table 1.

The precursor hHb had a small D_{eff} of $5.9 \pm 0.1 \text{ nm}$ and an estimated M_{W} of $64.5 \pm 0.0 \text{ kDa}$. Upon polymerization, T-state PolyhHb₁₀₀ exhibited a substantial increase in D_{eff} ($44.0 \pm 5.2 \text{ nm}$) and M_{W} ($2034 \pm 169 \text{ kDa}$), with both significantly higher than the precursor hHb ($p < 0.0001$). As the mass fraction of HSA increased in the Poly(hHb:HSA) conjugates, both the D_{eff} and M_{W} showed a decreasing trend. T-state Poly(hHb₇₅:HSA₂₅) had a D_{eff} of $29.6 \pm 2.3 \text{ nm}$ and M_{W} of $2024 \pm 262 \text{ kDa}$, while T-state Poly(hHb₅₀:HSA₅₀) further decreased to $21.5 \pm 1.9 \text{ nm}$ and $875 \pm 84 \text{ kDa}$. These decreases were statistically significant compared to T-state PolyhHb₁₀₀ ($p < 0.0001$ for both D_{eff} and M_{W}). Similarly, T-state Poly(hHb₂₅:HSA₇₅) showed a reduced D_{eff} ($14.5 \pm 1.1 \text{ nm}$) and M_{W} ($466 \pm 56 \text{ kDa}$), still significantly different from both the precursor hHb and T-state PolyhHb₁₀₀ ($p < 0.0001$ for most comparisons, except D_{eff} $p = 0.0608$ versus hHb). Interestingly, PolyHSA₁₀₀ had a D_{eff} of $24.6 \pm 5.8 \text{ nm}$ and a bimodal M_{W} distribution, with a high M_{W} fraction at $3270 \pm 14 \text{ kDa}$ and a low M_{W} fraction at $486 \pm 42 \text{ kDa}$. While its D_{eff} was lower than PolyhHb₁₀₀, the M_{W} of the high M_{W} fraction was even greater, suggesting higher order structures. Comparisons with the precursor hHb and other hHb:HSA copolymers were statistically significant ($p < 0.0001$ for most comparisons).

Upon polymerization, T-state PolyhHb₁₀₀ in the current study demonstrated a marked increase in $D_{\text{eff}} = 44.0 \pm 5.2 \text{ nm}$, aligning closely with the benchtop scale 30:1 T-state PolyhHb previously reported by our lab ($38.7 \pm 1.4 \text{ nm}$).⁵⁹ However, this value is larger than that observed for the pilot-scale 30:1 T-state PolyhHb ($26.3 \pm 5.1 \text{ nm}$),³⁹ likely due to improved mixing and reaction conditions in the pilot-scale reactor compared to the benchtop setup. In contrast, T-state Poly(hHb₇₅:HSA₂₅) showed a smaller D_{eff} of $29.6 \pm 2.3 \text{ nm}$. This reduction in size compared to T-state PolyhHb₁₀₀ may be

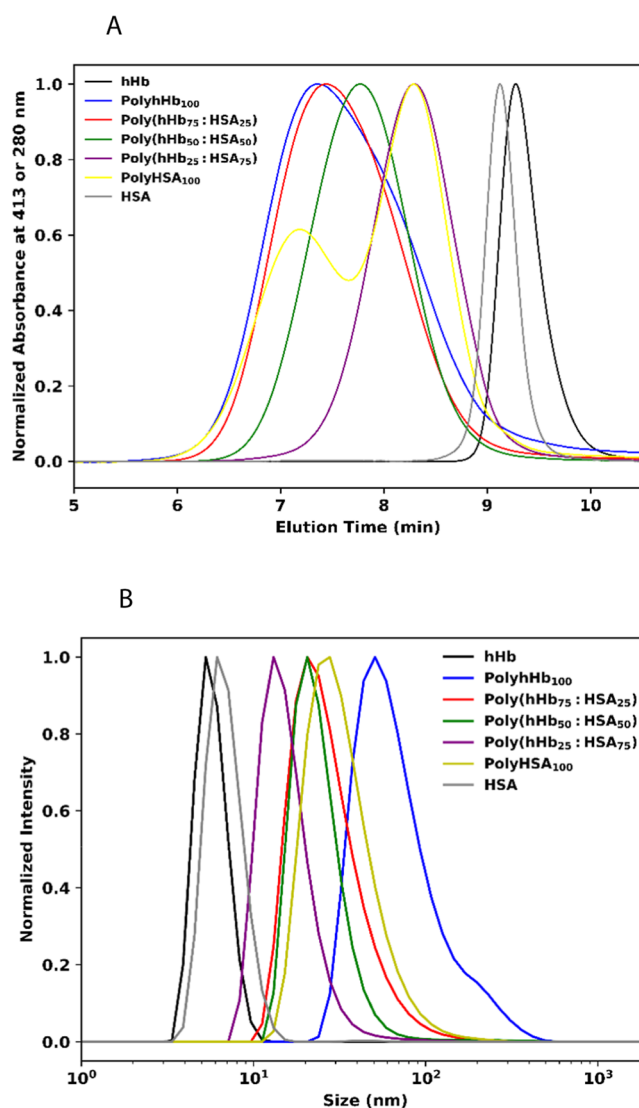


Figure 4. Size distribution and SEC-HPLC elution profiles of precursor hHb, T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) conjugates, and PolyHSA₁₀₀. (A) SEC-HPLC chromatograms were monitored at 413 nm for heme-containing proteins and 280 nm for total protein content from representative batches. (B) DLS spectra for the same samples, showing particle size distributions.

due to competitive cross-linking between the hHb and HSA molecules during copolymerization, which could limit the extent of hHb polymerization when both proteins are present. However, T-state Poly(hHb₇₅:HSA₂₅) achieved a comparable average yield to T-state PolyhHb₁₀₀. This suggests that while some competition for cross-linking may occur between hHb and HSA molecules, it does not substantially impact overall production yield at this mass fraction. In contrast, formulations containing more than 25% HSA mass fraction showed a marked decline in yield, indicating that T-state Poly(hHb₇₅:HSA₂₅) represents the optimal formulation in terms of maximizing production efficiency.

As the HSA mass fraction increased across the T-state Poly(hHb:HSA) conjugates, both D_{eff} and M_{W} followed a decreasing trend. This pattern suggests that the efficiency of cross-linking varies with protein composition. Specifically, the use of a 30:1 glutaraldehyde-to-hHb molar ratio is optimal for hHb polymerization, as established in our previous work.^{38,59}

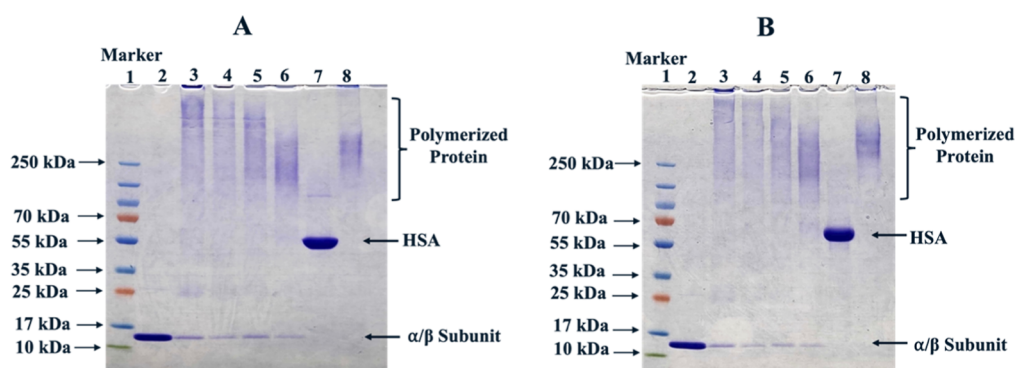


Figure 5. SDS-PAGE analysis of the precursor hHb, T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) conjugates, HSA, and PolyHSA₁₀₀ under nonreducing (A) and reducing conditions (B). Lane 1: M_W marker; Lane 2: precursor hHb; Lane 3: T-state PolyhHb₁₀₀; Lane 4: T-state Poly(hHb₇₅:HSA₂₅); Lane 5: T-state Poly(hHb₅₀:HSA₅₀); Lane 6: T-state Poly(hHb₂₅:HSA₇₅); Lane 7: precursor HSA; and Lane 8: PolyHSA₁₀₀.

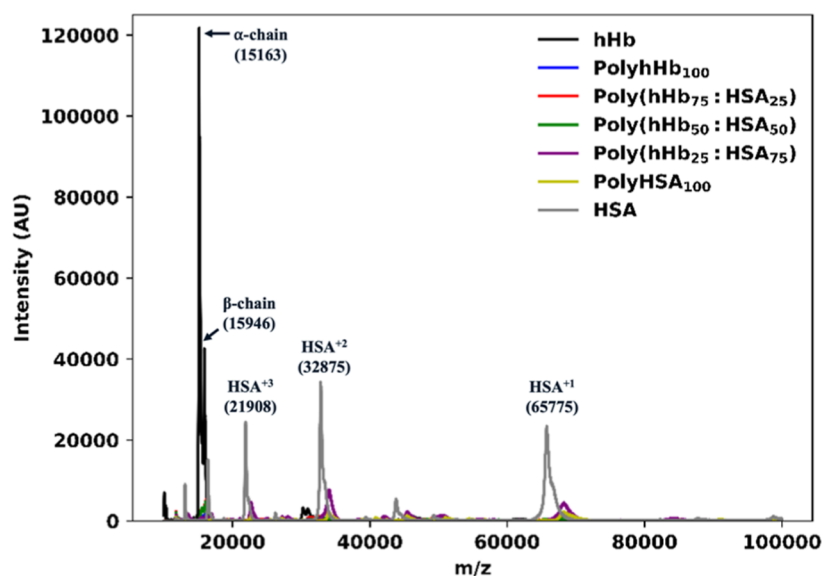


Figure 6. MALDI-TOF mass spectra for representative batches of the precursor hHb, T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) conjugates, precursor HSA, and PolyHSA₁₀₀.

Notably, the PolyHSA₁₀₀ formulation in this study exhibited a bimodal M_W profile, consisting of a high and low M_W fraction. Although high M_W polymers were present, the total yield was minimal (2%), suggesting that most of the product had a M_W below 500 kDa and was eliminated during purification over the 500 kDa TFF filter. These findings indicate that the 30:1 glutaraldehyde-to-protein ratio, which is effective for hHb polymerization, is insufficient for HSA polymerization. In contrast, efficient polymerization of HSA to achieve higher product yield has required higher cross-linker:protein ratios (e.g., 43:1 and 60:1 glutaraldehyde to HSA molar ratio), as reported in earlier studies from our lab.⁶⁰

T-state PolyhHb₁₀₀ and Poly(hHb₇₅:HSA₂₅) exhibited high M_W ~2000 kDa, while increasing HSA mass fraction in the hHb:HSA copolymers led to a progressive decline, Poly(hHb₅₀:HSA₅₀) decreased to ~800 kDa and Poly(hHb₂₅:HSA₇₅) to ~500 kDa. Despite these variations, all formulations retained high M_W and showed no detectable free tetrameric hHb after 500 kDa TFF filtration (Figure 4A,B), which is important for reducing risks such as NO scavenging, vasoconstriction, and systemic hypertension.⁷⁵ The absence of cell-free hHb and sustained M_W s above 500 kDa in T-state Poly(hHb₁₀₀) and Poly(hHb₇₅:HSA₂₅) suggest a lower

potential for these adverse side-effects. Additionally, higher M_W HBOCs are associated with prolonged circulation time, as observed with products ranging from Hemolink (192 kDa, 12–15 h) to HBOC-201 (576 kDa, 20 h).^{70,76} Although the current ~2000 kDa formulations are smaller than heavily cross-linked HBOCs 50:1 T- and 40:1 R-state PolyhHb and ZL-HbBv (16–42 MDa),^{47,65,77} they are larger than cross-linked hHb (O-R-polyHbA₀, 64–600 kDa),³⁴ oxyglobin (87–502 kDa),⁷¹ hemopure (250 kDa), and PolyHeme (150 kDa), the latter two of which contain up to 10% unmodified Hb.^{11,78} Thus, T-state PolyhHb₁₀₀ and Poly(hHb₇₅:HSA₂₅) are expected to offer prolonged circulation in the bloodstream.

3.7. SDS-PAGE. SDS-PAGE analysis of the precursor hHb, T-state PolyhHb₁₀₀, various T-state Poly(hHb:HSA) conjugates, HSA, and PolyHSA₁₀₀ was performed under both nonreducing and reducing conditions, as depicted in Figure 5A,B. Under both electrophoretic conditions, the precursor hHb exhibited a strong band at approximately 16 kDa, corresponding to the expected M_W of the α - and β -globin subunits. Similarly, HSA showed a prominent band between 55 and 70 kDa, consistent with its expected M_W (~67 kDa), as indicated by comparison with the protein ladder in lane 1. In contrast, T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) con-

jugates, and PolyHSA₁₀₀ samples exhibited broad, smeared bands in the high M_w region, indicative of the presence of polymeric species, a characteristic feature of PolyhHb and PolyHSA as described in the literature.^{60,79} All polymeric samples, including the PolyhHb₁₀₀ and Poly(hHb:HSA) conjugates, demonstrated predominant bands above 250 kDa. Only faint bands near 16 kDa and 30 kDa were observed, which correspond to free α - and β -subunits and dimeric species ($\alpha_2/\beta_2/\alpha\beta$), respectively. Collectively, these findings demonstrate that employing a 30:1 molar ratio of glutaraldehyde to hHb and HSA effectively promoted the formation of HMW polymeric species. Furthermore, the use of TFF efficiently removed unpolymerized, LMW components (<500 kDa), resulting in a purified population of cross-linked protein polymers.

3.8. MALDI-TOF Mass Spectrometry. MALDI-TOF mass spectrometry analysis of the precursor hHb, T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) conjugates, HSA, and PolyHSA₁₀₀ are presented in Figure 6. The spectrum for the precursor hHb displays two prominent peaks at 15,163 and 15,946 m/z , which correspond to the α - and β -globin subunits, respectively. In contrast, these characteristic peaks are absent in the spectra for T-state PolyhHb₁₀₀ and all T-state Poly(hHb:HSA) conjugates, indicating that no unpolymerized hHb was present in those materials. The precursor HSA spectrum exhibits three major peaks at 65,775 m/z , 32,875 m/z , and 21,908 m/z , corresponding to the $[M + H]^+$, $[M + H]^+$, and $[M + H]^+$ charge states, respectively, with the monomer peak aligning closely with the expected ~ 67 kDa M_w . No corresponding HSA peaks are observed for PolyHSA₁₀₀, suggesting complete polymerization with no free has present in the material. Overall, the MALDI-TOF spectra confirm that T-state PolyhHb₁₀₀, all T-state Poly(hHb:HSA) conjugates, and PolyHSA₁₀₀ lack significant levels of α/β -globin subunits or HSA in solution.

3.9. Circular Dichroism Spectroscopy. The influence of glutaraldehyde copolymerization with HSA on the structural integrity of hHb was evaluated using CD spectroscopy in both the far-UV (190–260 nm) and near-UV (250–500 nm) spectral regions to assess secondary and tertiary structural changes, respectively (Figure 7A,B). In the far-UV region, the CD spectra of the precursor hHb, T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) conjugates, as well as native HSA and PolyHSA₁₀₀, displayed characteristic α -helical features, with distinct negative bands near 208 and 222 nm. The precursor hHb showed the highest molar ellipticity at 222 nm ($-1.37 \times 10^7 \pm 602,330$ deg·cm²/dmol), indicating a well-preserved α -helical structure. Upon copolymerization, all hHb-containing formulations exhibited a decrease in molar ellipticity at 222 nm. For example, T-state PolyhHb₁₀₀ and T-state Poly(hHb₇₅:HSA₂₅) showed slightly less negative values ($-1.22 \times 10^7 \pm 1,410,383$ deg·cm²/dmol and $-1.25 \times 10^7 \pm 1,770,506$ deg·cm²/dmol respectively), while T-state Poly(hHb₅₀:HSA₅₀) and T-state Poly(hHb₂₅:HSA₇₅) continued this trend ($-1.21 \times 10^7 \pm 813,009$ deg·cm²/dmol and $-1.21 \times 10^7 \pm 903,256$ deg·cm²/dmol, respectively). Interestingly, the ellipticity of PolyHSA₁₀₀ ($-1.30 \times 10^7 \pm 1,338,624$ deg·cm²/dmol) was slightly more negative than the hHb:HSA copolymers, while the precursor HSA ($-1.27 \times 10^7 \pm 315,657$ deg·cm²/dmol) also demonstrated a relatively high helical content. However, statistical comparisons revealed no significant differences among the formulations (all p -values >0.36), indicating that the secondary structure of the

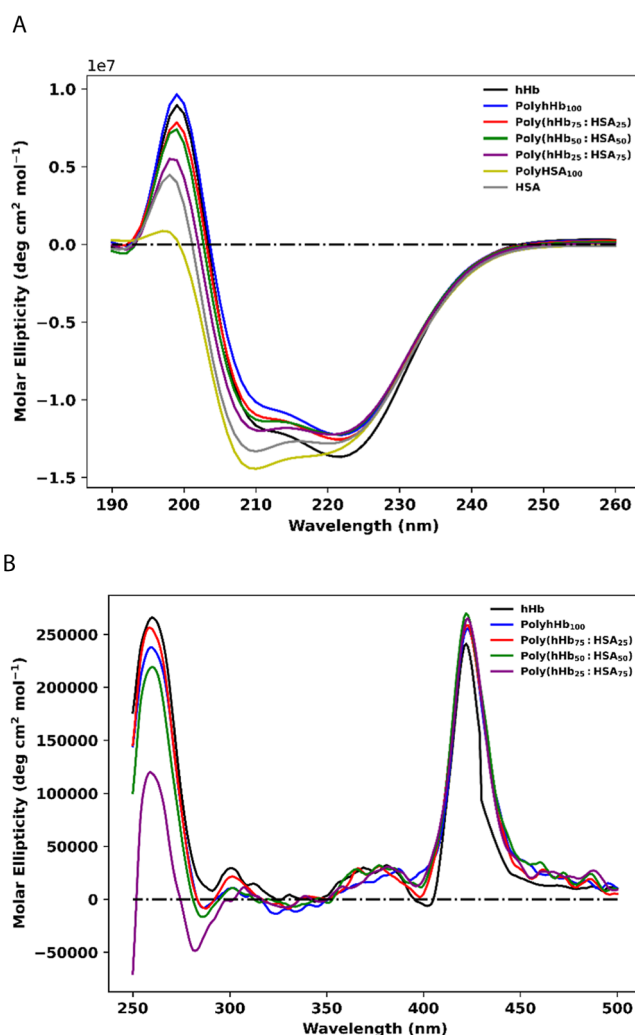


Figure 7. Circular dichroism spectra of the precursor hHb, T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) conjugates, precursor HSA, and PolyHSA₁₀₀. (A) Far-UV CD (190–260 nm) and (B) near-UV CD (250–500 nm) spectra were recorded at room temperature in 150 mM PBS (pH 7.4) using a 0.1 cm path length cuvette. The data reflects the average of 3 independent batches of all the materials.

hHb:HSA copolymers remain largely consistent despite variations in HSA mass fraction.

Near-UV CD spectroscopy analysis at wavelengths 422 and 260 nm reveal distinct trends in molar ellipticity, emphasizing the effects of polymerization and increasing HSA incorporation into T-state PolyhHb constructs. At 422 nm, which reflects the heme environment within the globin structure, molar ellipticity values exhibited a modest upward trend from 241,138 $\pm 16,873$ deg·cm²·dmol⁻¹ for the precursor hHb to 269,901 $\pm 14,378$ deg·cm²·dmol⁻¹ for T-state PolyhHb₂₅:HSA₇₅. Despite this increase, statistical analysis showed no significant differences among the groups (all p -values >0.05), suggesting that the observed changes in the tertiary structure near the heme moiety were not statistically meaningful. In contrast, at 260 nm, which is more responsive to base stacking interactions and aromatic side-chain environments, the changes in molar ellipticity were both more pronounced and statistically significant. The precursor hHb exhibited the highest value at 266,077 $\pm 10,479$ deg·cm²·dmol⁻¹, which dropped sharply to 118,779 $\pm 15,871$ deg·cm²·dmol⁻¹ for T-state Poly(hHb₂₅:HSA₇₅), indicating a notable alteration in the tertiary

structure. While some comparisons, such as precursor hHb versus T-state PolyhHb₁₀₀ ($p = 0.0554$) and precursor hHb versus T-state Poly(hHb₇₅:HSA₂₅) ($p = 0.7323$), were not statistically significant, several others revealed highly significant differences. In particular, comparisons such as precursor hHb versus T-state Poly(hHb₂₅:HSA₇₅) and PolyhHb₁₀₀ versus T-state Poly(hHb₂₅:HSA₇₅) produced p -values <0.0001 , reflecting substantial structural rearrangements of the chromophore environment with increased HSA incorporation. These findings suggest that while polymerization and HSA copolymerization have minimal impact on the heme environment (as observed at 422 nm), they significantly alter other tertiary structural features, particularly those affecting the 260 nm region.

3.10. Thermal Stability. The thermal stability of the precursor hHb, T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) conjugates, precursor HSA, and PolyHSA₁₀₀ was analyzed using CD spectroscopy (Figure 8). By tracking changes in the

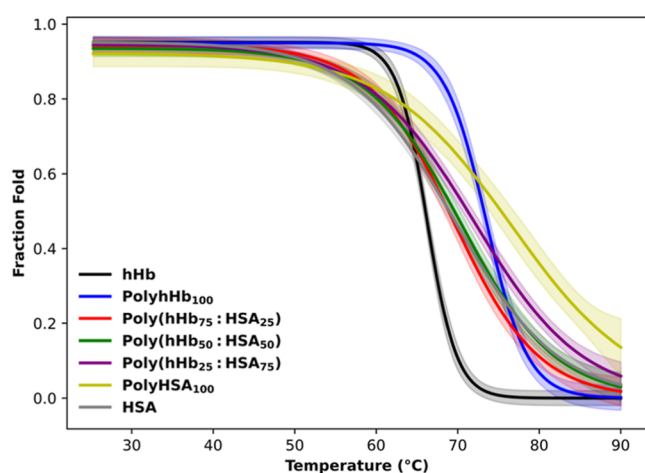


Figure 8. Thermal stability profiles of the precursor hHb, T-state PolyhHb₁₀₀, T-state Poly(hHb:HSA) conjugates, precursor HSA, and PolyHSA₁₀₀. The unfolding of protein secondary structure was tracked by changes in the molar ellipticity at 222 nm using far-UV CD as the temperature was increased. Normalized ellipticity values at 222 nm were plotted against temperature. The data represent the average of 3 batches with standard deviations indicated by shaded regions corresponding to each curve's color.

normalized molar ellipticity at 222 nm across a temperature gradient (25–90 °C), thermal unfolding profiles were constructed, and the melting temperature (T_m) was identified as the midpoint of protein denaturation. T_m values, which serve as indicators of thermal stability, are presented in Table 1. The precursor hHb exhibited a T_m of 66.2 ± 0.4 °C, in agreement with prior findings.⁸⁰ Polymerization led to a notable increase in stability, with T-state PolyhHb₁₀₀ reaching a T_m of 73.5 ± 0.2 °C, a statistically significant improvement over the precursor hHb ($p = 0.0004$), highlighting the stabilizing effect of inter and intramolecular cross-linking. However, no additional enhancement was observed upon incorporation of has into the copolymer. Among the hHb:HSA conjugates, T-state Poly(hHb₂₅:HSA₇₅) showed the highest T_m of 71.9 ± 0.8 °C, significantly greater than that of precursor hHb ($p < 0.0001$), indicating that higher HSA content contributes to greater thermal resistance. T-state Poly(hHb₅₀:HSA₅₀) and T-state Poly(hHb₇₅:HSA₂₅) also demonstrated increased T_m values (70.9 ± 2.6 °C and 69.5 ± 2.4 °C, respectively),

although their statistical significance decreased with lower HSA content ($p = 0.0212$ and $p = 0.1677$). PolyHSA₁₀₀ exhibited the highest thermal stability overall, with a T_m of 77.8 ± 0.5 °C, significantly surpassing that of the precursor HSA (70.3 ± 0.3 °C, $p = 0.0004$), confirming that polymerization alone enhances protein stability. In summary, polymerization plays a more significant role in copolymer stability than HSA incorporation to contribute meaningfully in improving the thermal stability of polymerized biomaterials.

3.11. Auto-Oxidation Kinetics. The auto-oxidation kinetics of the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates were evaluated following established protocols.⁴⁶ Representative kinetic traces are shown in Figure 9, while Table 1 summarizes the

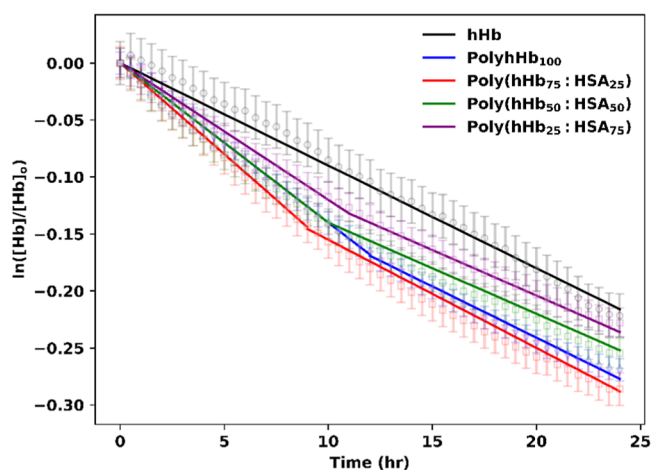


Figure 9. Auto-oxidation kinetics of the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates at 0.775 mM (heme basis). Reactions were monitored in 150 mM PBS (pH 7.4) at 37 °C. The normalized data was fitted using a two-phase linear regression model. The results represent the averages from 3 independent batches of material along with the standard deviation.

corresponding rate constants (k_{ox}). The precursor hHb followed a single-phase oxidation profile, whereas T-state PolyhHb₁₀₀ exhibited biphasic behavior, likely indicating differential oxidation of the α - and β -globin chains, as reported previously.^{59,81} Among all samples, the precursor hHb showed the slowest oxidation rate at 0.010 ± 0.001 h⁻¹, aligning with prior literature.⁴⁶ Upon polymerization, T-state PolyhHb₁₀₀ demonstrated a significant increase in the fast-phase oxidation rate to 0.014 ± 0.001 h⁻¹ ($p = 0.0229$ versus hHb), consistent with earlier findings indicating that T-state polymerization accelerates auto-oxidation.^{46,59} The two-phase pattern observed in the auto-oxidation of PolyhHb could be due to the α -chains oxidizing more rapidly than the β -chains.⁸² Among the T-state Poly(hHb:HSA) conjugates, the fast-phase k_{ox} values ranged from 0.012 ± 0.001 h⁻¹ for T-state Poly(hHb₂₅:HSA₇₅) to 0.015 ± 0.002 h⁻¹ for T-state Poly(hHb₅₀:HSA₅₀). Statistical analysis showed that T-state Poly(hHb₇₅:HSA₂₅) ($p = 0.0055$) and T-state Poly(hHb₅₀:HSA₅₀) ($p = 0.0229$) had significantly higher oxidation rates compared to the precursor hHb. However, statistical comparisons between the polymerized samples showed no significant differences in fast-phase auto-oxidation rate constants for example, between T-state PolyhHb₁₀₀ and Poly(hHb₇₅:HSA₂₅) ($p = 0.8714$), or between T-state PolyhHb₁₀₀ and Poly(hHb₂₅:HSA₇₅) ($p = 0.2498$). These findings suggest that once polymerization

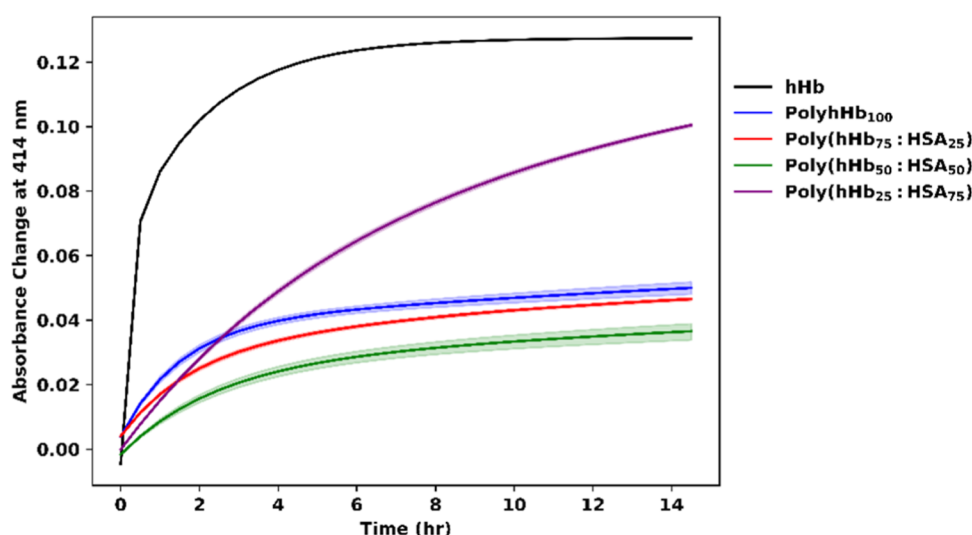


Figure 10. Kinetics of heme transfer from the methHb forms of the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates to Hpx. Reactions were carried out at 37 °C with 2 μ M HBOC (Hb tetramer basis), and the absorbance monitored at 414 nm. Rate constants were determined by fitting the data to a double exponential model. The data shown are averages from 3 batches, with standard deviations indicated by shaded regions corresponding to each curve's color.

occurs, the proportion of HSA incorporated has minimal influence on the fast-phase oxidation kinetics. In contrast, the slow-phase auto-oxidation detected only in the polymerized constructs was relatively consistent across all formulations, with rate constants between $0.008 \pm 0.001 \text{ h}^{-1}$ and $0.009 \pm 0.001 \text{ h}^{-1}$, and no significant differences observed among the different Poly(hHb:HSA) conjugates ($p > 0.3$).

The T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates display elevated auto-oxidation rate constants compared to the precursor hHb. This increase in oxidation is a major obstacle in the development of HBOCs, as it compromises oxygen carrying capacity and can lead to cellular toxicity due to the release of heme from the oxidized hHb construct. The likely cause is chemical modification, specifically glutaraldehyde cross-linking which may interfere with normal interactions between the α - and β -subunits.^{82,83} Furthermore, it has been shown that partial oxygen saturation can accelerate auto-oxidation in myoglobin. At room temperature and atmospheric oxygen partial pressure (148 mmHg), T-state PolyhHb is only partially saturated with oxygen (50–70%), which could contribute to the enhanced oxidation.^{84,85} Despite this oxidation susceptibility, T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates synthesized in this study demonstrated much lower auto-oxidation rate constants than those reported for other HBOCs, such as Hemopure ($0.220 \pm 0.020 \text{ h}^{-1}$) and PolyHeme ($0.260 \pm 0.010 \text{ h}^{-1}$).⁶⁴ A plausible explanation for this improved oxidative stability is the presence of residual antioxidant enzymes such as catalase (CAT) originating from RBCs and retained during the purification of the precursor hHb using a 50 kDa hollow fiber TFF system.

3.12. Heme Release Kinetics. The heme release kinetics of the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates was evaluated following established protocols.⁴⁶ Representative kinetic traces are shown in Figure 10, while Table 1 summarizes the corresponding rate constants ($k_{\text{heme release}}$) expressed in units of h^{-1} . The precursor hHb displayed a biphasic heme release pattern, where the rapid phase corresponds to heme dissociation from the β -globin chains, while the slower phase is attributed to heme release

from the α -globin chains, as previously described in the literature.⁸⁶ Analysis of the heme release kinetics reveals a clear trend in both the fast (k_{fast}) and slow (k_{slow}) heme release rates for T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates formulations. The precursor hHb exhibited the highest fast heme release rate constant ($k_{\text{fast}} = 5.08 \pm 0.32 \text{ h}^{-1}$), which significantly decreased upon polymerization. T-state PolyhHb₁₀₀ showed a markedly reduced rate ($0.63 \pm 0.04 \text{ h}^{-1}$), with further decreases observed as the HSA content increased: $0.56 \pm 0.03 \text{ h}^{-1}$ in T-state Poly(hHb₇₅:HSA₂₅), $0.41 \pm 0.03 \text{ h}^{-1}$ in T-state Poly(hHb₅₀:HSA₅₀), and $0.18 \pm 0.01 \text{ h}^{-1}$ in T-state Poly(hHb₂₅:HSA₇₅). These reductions were statistically significant when comparing hHb with T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) formulations ($p < 0.0001$ for all comparisons). Among the copolymers, significant differences in k_{fast} was observed between T-state PolyhHb₁₀₀ and T-state Poly(hHb₂₅:HSA₇₅) ($p = 0.022$), while T-state PolyhHb₁₀₀, T-state poly(hHb₇₅:HSA₂₅), and T-state Poly(hHb₅₀:HSA₅₀) were all not significantly different from each other. This result suggests that the incorporation of HSA into the copolymer does not affect fast-phase heme loss kinetics until HSA becomes the dominant component in the copolymer. Similarly, the slow-phase heme release rate constant (k_{slow}) showed a marked decrease across the polymeric formulations. The precursor hHb exhibited the highest k_{slow} ($0.48 \pm 0.003 \text{ h}^{-1}$), significantly greater than all T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates ($p < 0.0001$). The k_{slow} values for the polymeric variants were, PolyhHb₁₀₀: $0.03 \pm 0.005 \text{ h}^{-1}$, Poly(hHb₇₅:HSA₂₅): $0.08 \pm 0.001 \text{ h}^{-1}$, Poly(hHb₅₀:HSA₅₀): $0.03 \pm 0.01 \text{ h}^{-1}$, and Poly(hHb₂₅:HSA₇₅): $0.04 \pm 0.001 \text{ h}^{-1}$. All pairwise comparisons between hHb and each copolymer were statistically significant ($p < 0.0001$).

One key challenge in the development of HBOCs is minimizing the rapid release of free heme into circulation, as free heme can function as a pro-oxidant, promoting lipid and protein oxidation via reactions with reactive oxygen species.⁸⁷ Additionally, heme loss destabilizes hHb, leading to unfolding and precipitation of the apoprotein leading to the formation of Heinz bodies.^{88,89} Therefore, when modifying hHb such as

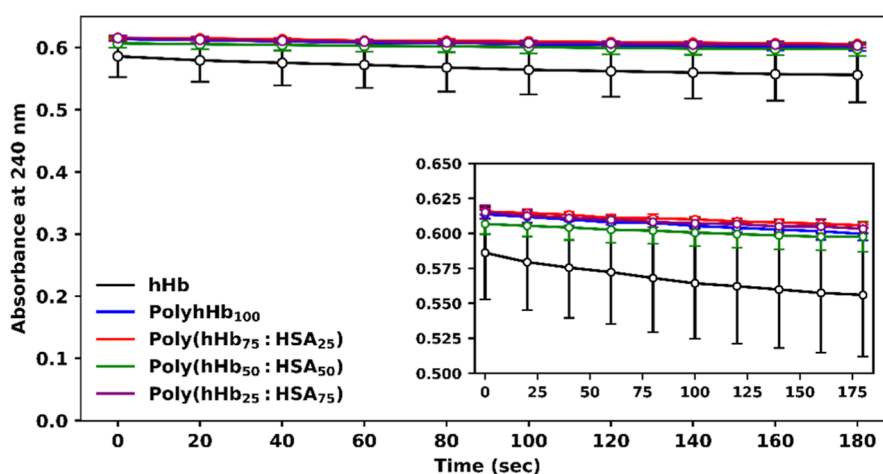


Figure 11. CAT activity in the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates assessed by the decomposition of hydrogen peroxide at 240 nm using UV–visible spectroscopy. Experiments were performed in 150 mM PBS (pH 7.4) at room temperature.

through glutaraldehyde polymerization and cross-linking with HSA, it is critical to assess the rate of heme release from both PolyhHb and Poly(Hb:HSA) conjugates to ensure HBOC stability and efficacy. Heme transfer studies from the oxidized T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates to Hpx demonstrate that these polymerized forms retain heme significantly more effectively than the precursor hHb. This enhanced retention of heme is likely due to extensive cross-linking between the α - and β -globin chains, which may protect internal Hb subunits from interacting with Hpx, thereby limiting heme release to only those molecules exposed on the polymer surface. Notably, both the fast and slow phases of heme release decrease substantially with the incorporation of HSA, an added benefit likely due to HSA binding the released heme before its transfer to Hpx, effectively sequestering it and further reducing its bioavailability.

3.13. Catalase Activity Assay. CAT activity in the precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates was measured using previously described methods.⁴⁶ Representative kinetic profiles are displayed in Figure 11, and the corresponding enzymatic activities, reported in units of U/mL, are summarized in Table 1. Absolute activities, expressed in U/mL of solution, and specific activities, expressed in U/mg protein, are both reported. CAT concentrations in the samples were determined using a standard calibration curve.

CAT activity was highest in the precursor hHb group (6.67 ± 2.40 U/mL) and significantly decreased following polymerization. T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates showed markedly lower CAT activity compared to the precursor hHb. T-state PolyhHb₁₀₀ exhibited a significant reduction in activity (3.13 ± 0.42 U/mL, $p = 0.0246$), T-state Poly(hHb₇₅:HSA₂₅) (2.20 ± 0.21 U/mL, $p = 0.0056$), T-state Poly(hHb₅₀:HSA₅₀) (2.02 ± 0.78 U/mL, $p = 0.0043$), and T-state Poly(hHb₂₅:HSA₇₅) (2.66 ± 0.38 U/mL, $p = 0.0115$), when compared with the precursor hHb. However, no statistically significant differences in CAT activity were observed among the different polymerized formulations. For example, T-state PolyhHb₁₀₀ did not differ significantly from T-state Poly(hHb₇₅:HSA₂₅) ($p = 0.8571$), T-state Poly(hHb₅₀:HSA₅₀) ($p = 0.7677$) or T-state Poly(hHb₂₅:HSA₇₅) ($p = 0.9859$). Similarly, comparisons between T-state Poly(hHb₇₅:HSA₂₅), T-state Poly(hHb₅₀:HSA₅₀), and

T-state Poly(hHb₂₅:HSA₇₅) also showed no significant differences (p -values well above 0.05). However, these data show that while the absolute activities vary due to differing concentrations, the specific activities are in a similar range (0.021–0.033 Units/mg). Thus, the intrinsic catalase activity is largely retained across the hHb-HSA conjugates.

This outcome is consistent with expectations, as the precursor hHb was filtered using TFF within a 50–500 kDa M_w range, which retains CAT (~ 240 kDa). In contrast, both the T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates underwent TFF purification with a 500 kDa cutoff after polymerization. Since the cross-linking process was designed to primarily modify hHb, high M_w PolyhHb was retained, while unmodified or minimally polymerized CAT below 500 kDa in M_w was removed during filtration. Furthermore, increasing the mass fraction of HSA in the polymerization reaction reduced the amount of starting hHb, and therefore the initial CAT content. Despite this, CAT activity in T-state Poly(hHb:HSA) conjugates remained similar to that in T-state PolyhHb₁₀₀. This suggests that HSA may confer a protective effect, helping to preserve CAT activity, an observation also supported by previous findings.⁹⁰ An inverse correlation was observed between CAT activity and auto-oxidation rate constants: the precursor hHb, with the highest CAT activity, exhibited the slowest auto-oxidation rate, while all polymerized forms showed reduced CAT activity and increased auto-oxidation. Although CAT contributes to oxidative stability, auto-oxidation is influenced by multiple factors, as discussed in the auto-oxidation section. Notably, despite reduced CAT activity, T-state PolyhHb₁₀₀ and Poly(hHb:HSA) conjugates synthesized in this study exhibited lower auto-oxidation rates than commercial HBOCs such as Hemopure and PolyHeme.⁶⁴ These findings suggest that while polymerization diminishes CAT activity, HSA incorporation does not significantly impact this loss in activity, and the resulting T-state Poly(hHb:HSA) conjugates still retain antioxidant properties, making them promising HBOC candidates.

3.14. Zeta Potential. The zeta potential values of T-state PolyhHb₁₀₀ and T-state Poly(hHb:HSA) conjugates are reported in Table 1. The precursor hHb and HSA exhibited a surface charge (-0.6 ± 0.4 mV and -15.0 ± 1.7 mV, respectively), whereas polymerization markedly increased the negative surface potential. PolyhHb₁₀₀ showed a significantly

lower zeta potential (-18.8 ± 1.8 mV; $p < 0.0001$ vs hHb). The incorporation of HSA into the copolymer progressively increased the negative charge: Poly(hHb₇₅:HSA₂₅) = -23.2 ± 1.3 mV ($p < 0.0001$ vs hHb), Poly(hHb₅₀:HSA₅₀) = -27.0 ± 1.7 mV ($p < 0.0001$ vs hHb), and Poly(hHb₂₅:HSA₇₅) = -26.7 ± 1.9 mV ($p < 0.0001$ vs hHb). Comparisons among Poly(hHb:HSA) conjugates showed no significant differences between Poly(hHb₂₅:HSA₇₅) and Poly(hHb₅₀:HSA₅₀) ($p = 0.9999$), or between Poly(hHb₇₅:HSA₂₅) and Poly(hHb₂₅:HSA₇₅) ($p = 0.0951$). However, PolyhHb₁₀₀ remained significantly different from all copolymers (e.g., $p = 0.0002$ vs Poly(hHb₅₀:HSA₅₀); $p = 0.0003$ vs Poly(hHb₂₅:HSA₇₅)). Overall, increasing HSA content led to a more negative zeta potential up to 50% mass fraction incorporation, after which the value plateaued, indicating saturation of the copolymer surface charge. This negative charge is expected to reduce the potential likelihood of Poly(hHb:HSA) conjugates adhering to the vascular endothelium in vivo or other plasma proteins in the circulation.

3.15. RBC Aggregation, Viscosity and Colloid Osmotic Pressure. The Poly(hHb:HSA) copolymers significantly increased RBC aggregation compared to acellular Hb as shown in Figure 12 and Table 2.

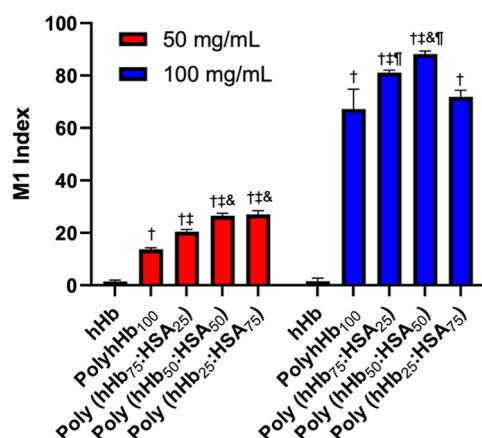


Figure 12. RBC Aggregation. Utilizing a Myrenne aggregometer (Myrenne GmbH, Roetgen, Germany) to assess the effects of hHb:HSA copolymers and their effects on RBC aggregation at low shear rates, measured using the M1 index over an integration time of 10 s. The aggregation study was performed using a 1:2 (vol:vol) ratio of HBOC to whole blood, with five individual samples per copolymer formulation, and three measurements were taken per sample to ensure consistency (total volume per sample was 30 μ L) this was repeated across blood of four different blood samples of the same whole blood stock. † vs hHb, ‡ vs PolyhHb₁₀₀, § vs Poly(hHb₇₅:HSA₂₅), and ¶ vs Poly(hHb₂₅:HSA₇₅).

All Poly(hHb:HSA) formulations produced significantly higher aggregation indices (M1) ($p < 0.0001$) at both 50 and 100 mg/mL concentrations. At 50 mg/mL, PolyhHb₁₀₀ exhibited significantly lower aggregation indices than all

other Poly(hHb:HSA) formulations ($p < 0.0001$). However, at 100 mg/mL, PolyhHb₁₀₀ still showed lower aggregation indices, though to a lesser extent, remaining significantly smaller than Poly(hHb₇₅:HSA₂₅) ($p < 0.01$) and Poly(hHb₅₀:HSA₅₀) ($p < 0.001$), with no significant difference compared to Poly(hHb₂₅:HSA₇₅). At both 50 and 100 mg/mL, Poly(hHb₇₅:HSA₂₅) exhibited significantly lower aggregation indices than Poly(hHb₅₀:HSA₅₀) ($p < 0.0001$) and Poly(hHb₂₅:HSA₇₅) ($p < 0.0001$). Finally, Poly(hHb₅₀:HSA₅₀) showed significantly higher aggregation indices than Poly(hHb₂₅:HSA₇₅) at 100 mg/mL ($p < 0.0001$), but not at 50 mg/mL. At 100 mg mL⁻¹, RBC aggregation further intensified across all groups, demonstrating a concentration-dependent response.

All solutions exhibited shear-thinning behavior, with viscosity (η) decreasing as the shear rate ($\dot{\gamma}$) increased (Figures 13, 14 and Table 3).

At matched $\dot{\gamma}$, Poly(hHb:HSA) formulations were consistently more viscous than hHb, and increasing total protein concentration from 50 mg mL⁻¹ to 100 mg mL⁻¹ produced higher viscosities in all copolymer formulations. Among them, Poly(hHb₅₀:HSA₅₀) displayed the largest separation between the two concentrations, indicating a strong sensitivity to concentration. In contrast, hHb showed minimal viscosity changes between 50 and 100 mg mL⁻¹, behaving nearly Newtonian across the tested range.

3.15.1. Concentration-Dependent Rheological Scaling. The viscosity scaling exponent α , derived from $\eta_{100}/\eta_{50} = 2^\alpha$, revealed clear concentration-dependent rheological behavior (Figure 15 and Table 4).

α is defined as $\alpha = \ln(\eta_{100}/\eta_{50})/\ln(100/50)$, where viscosities (η) were measured at 37 °C on a TA HR-2 using a 2° cone during a 1000→10→1000 s⁻¹ flow sweep (10 measurements per log step); values at each shear rate are the average of descending and ascending measurement. The dashed line marks $\alpha = 1$ (linear scaling: doubling concentration doubles viscosity). Solid lines are linear fits, $\alpha = a + s * \ln(\dot{\gamma})$, where the slope s maps to the difference in flow index $\Delta n = n_{100} - n_{50} = s * \ln(2)$ (positive $\Rightarrow$ the concentrated sample is less shear-thinning). Poly(hHb₅₀:HSA₅₀) shows the greatest α and strongest positive slope; hHb exhibits the smallest α with a negative slope.

All Poly(hHb:HSA) formulations displayed $\alpha > 1$, indicating superlinear viscosity scaling with concentration. Poly(hHb₅₀:HSA₅₀) showed the highest α values and the steepest positive slope in α vs $\ln \dot{\gamma}$, corresponding to a positive $\Delta n (=n_{100} - n_{50})$ and suggesting the concentrated solution was less shear-thinning than its dilute counterpart. Conversely, hHb exhibited $\alpha \approx 1$ and slightly negative slopes ($\Delta n < 0$), indicating reduced intermolecular interactions and a mild increase in shear-thinning at higher concentrations. These findings demonstrate that HSA incorporation enhances cohesive hydrodynamic interactions and concentration-dependent viscoelasticity within the copolymer network.

Table 2. RBC Aggregation Index Values (No Units)^a

protein concentration (mg/mL)	precursor hHb	polyhHb ₁₀₀	poly(hHb ₇₅ :HSA ₂₅)	poly(hHb ₅₀ :HSA ₅₀)	poly(hHb ₂₅ :HSA ₇₅)
50	1.42 $\pm$ 0.52	13.74 $\pm$ 0.52 ^b	20.46 $\pm$ 0.80 ^{b,c}	25.53 $\pm$ 0.93 ^{b,c,d}	27.03 $\pm$ 1.41 ^{b,c,d}
100	1.54 $\pm$ 1.17	67.24 $\pm$ 7.57 ^b	81.13 $\pm$ 0.94 ^{b,c}	88.24 $\pm$ 1.12 ^{b,c,d,e}	71.83 $\pm$ 2.58 ^{b,c,d}

^aAverage RBC aggregation (M, or index at stasis) values recorded. Mean $\pm$ standard deviation. ^bvs Hb. ^cvs PolyhHb₁₀₀. ^dvs Poly(hHb₇₅:HSA₂₅). ^evs Poly(hHb₂₅:HSA₇₅).

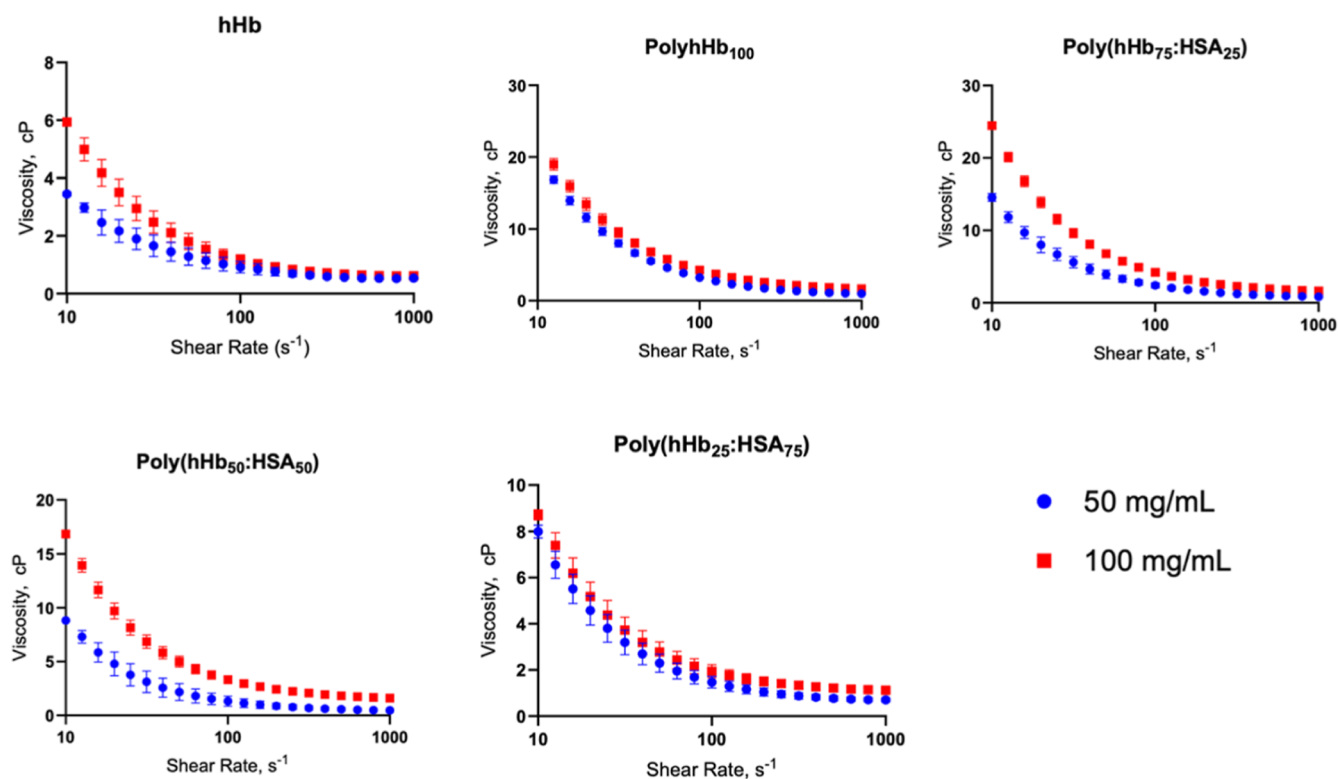


Figure 13. Viscosity–shear rate behavior of hHb and Poly(hHb:HSA) formulations at two concentrations. Flow sweeps were run on a TA Discovery HR-2 at 37 °C with a 2° cone ($R = 20$ mm; ~ 50 μ m truncation) using a $1000 \rightarrow 10 \rightarrow 1000$ s^{-1} protocol (log steps; 10 measurements per step). For each shear rate, values are the average of the descending and ascending measurements; error bars show within-step variability. Blue: 50 $mg\ mL^{-1}$; red: 100 $mg\ mL^{-1}$. All polymer formulations are shear-thinning (η decreases with $\dot{\gamma}$) and, at matched $\dot{\gamma}$, the 100 $mg\ mL^{-1}$ samples are generally more viscous than 50 $mg\ mL^{-1}$, with the largest separation in Poly(hHb₅₀:HSA₅₀). The precursor hHb shows minimal concentration dependence across the range.

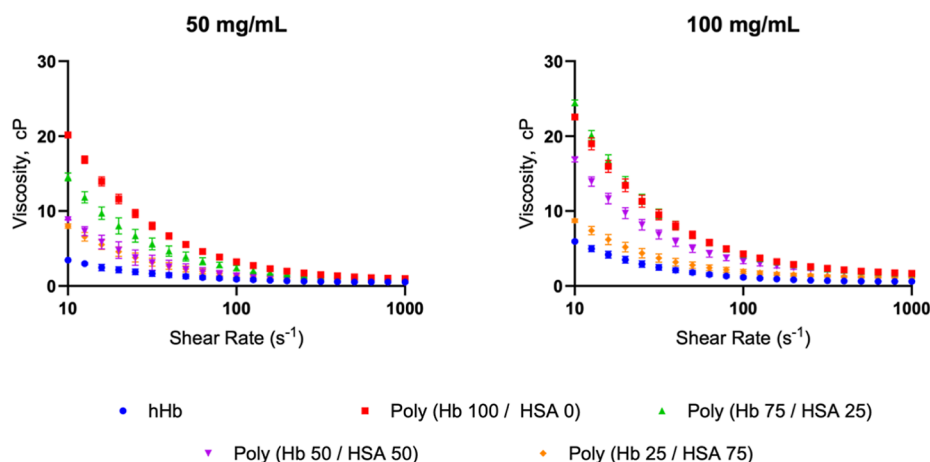


Figure 14. Flow Sweeps. Viscosity of different Poly(hHb:HSA) copolymers at (A) 50 mg/mL and (B) 100 mg/mL . Utilizing a TA Instruments Discovery HR-2 rheometer (New Castle, Delaware, United States), featuring an aluminum 40 mm flat plate, solutions were tested with a total solution volume of 0.63 mL. All samples were maintained at 37°C to mimic physiological conditions. To assess variations in fluid viscosity among the groups, a steady-state flow shear rate sweep test was conducted, spanning from 1 to 1000 s^{-1} , with shear rates increasing from maximum to minimum and then ascending.

3.15.2. Colloid Osmotic Pressure. Colloid osmotic pressure (COP) increased with the mass fraction of HSA in the copolymer mixture as shown in Figure 16 and Table 5.

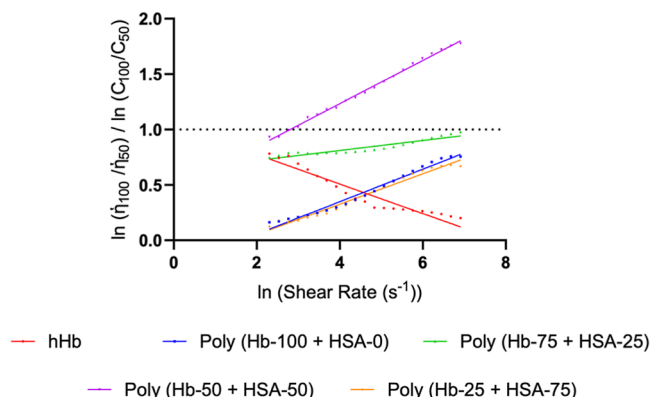
Acellular Hb exhibited the highest COP at all concentrations when compared to all Poly(hHb:HSA) formulations, at 10 mg/mL ($p < 0.01$), 50 mg/mL ($p < 0.0001$), and 100 mg/mL ($p < 0.0001$). Among the Poly(hHb:HSA) formulations, there were no significant differences at 10 mg/mL . However, at 50

and 100 mg/mL , PolyhHb₁₀₀ showed significantly lower COP than Poly(hHb₅₀:HSA₅₀) ($p < 0.05$) and Poly(hHb₂₅:HSA₇₅) ($p < 0.001$), but was not significantly different from Poly(hHb₇₅:HSA₂₅). Poly(hHb₇₅:HSA₂₅) exhibited significantly lower COP at 50 mg/mL compared to Poly(hHb₅₀:HSA₅₀) ($p < 0.01$) and Poly(hHb₂₅:HSA₇₅) ($p < 0.001$). At 100 mg/mL , Poly(hHb₇₅:HSA₂₅) remained significantly lower than Poly(hHb₂₅:HSA₇₅) ($p < 0.001$),

Table 3. Viscosity Values at Different Shear Rates (units = cP)^a

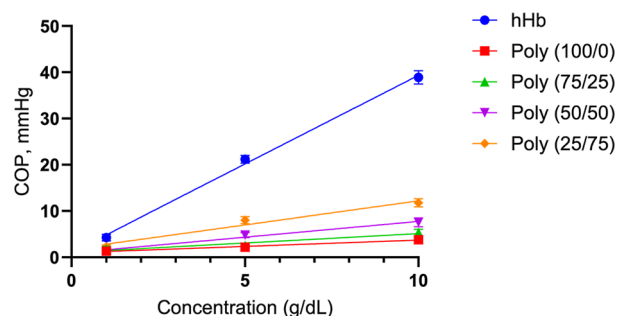
viscosity (cP)		precursor hHb	polyhHb ₁₀₀	poly(hHb ₇₅ :HSA ₂₅)	poly(hHb ₅₀ :HSA ₅₀)	poly(hHb ₂₅ :HSA ₇₅)
50 (mg/mL)	10 s ⁻¹	3.45 ± 0.07	20.2 ± 0.2	14.6 ± 0.5	8.83 ± 0.05	8.00 ± 0.28
	100 s ⁻¹	0.92 ± 0.20	3.24 ± 0.16	2.41 ± 0.33	1.32 ± 0.47	1.47 ± 0.25
	1000 s ⁻¹	0.54 ± 0.01	0.99 ± 0.05	0.83 ± 0.04	0.47 ± 0.07	0.70 ± 0.07
100 (mg/mL)	10 s ⁻¹	5.94 ± 0.12	22.6 ± 0.3	24.5 ± 0.4	16.9 ± 0.3	8.71 ± 0.22
	100 s ⁻¹	1.17 ± 0.17	4.29 ± 0.32	4.22 ± 0.26	3.31 ± 0.33	1.95 ± 0.28
	1000 s ⁻¹	0.62 ± 0.01	1.68 ± 0.01	1.64 ± 0.03	1.61 ± 0.09	1.12 ± 0.08

^aViscosity values of different Poly(hHb:HSA) co-polymers at 50 and 100 mg/mL. Utilizing a TA Instruments Discovery HR-2 rheometer (New Castle, Delaware, United States), featuring an aluminum 40 mm flat plate, solutions were tested with a total solution volume of 0.63 mL. All samples were maintained at 37 °C to mimic physiological conditions. To assess variations in fluid viscosity among the groups, a steady-state flow shear rate sweep test was conducted, spanning from 1 to 1000 s⁻¹, with shear rates increasing from minimum to maximum and then decreasing.

**Figure 15.** Concentration exponent (α) vs shear rate for hHb and Poly(hHb:HSA) copolymers.

with no significant difference from Poly(hHb₅₀:HSA₅₀). Finally, Poly(hHb₅₀:HSA₅₀) showed significantly lower COP than Poly(hHb₂₅:HSA₇₅) at both 50 mg/mL ($p < 0.01$) and 100 mg/mL ($p < 0.01$).

The present study demonstrates that Poly(hHb:HSA) formulations exhibit distinct hemorheological and COP that are strongly influenced by the mass ratio of hHb to HSA. Incorporating HSA into the copolymer significantly altered RBC aggregation, viscosity, and COP, revealing that balanced hHb:HSA mass ratios yield the most physiologically favorable characteristics. These findings highlight the critical role of copolymer composition in tuning both the biophysical and functional properties of HBOCs for intravascular compatibility and fluid resuscitation. RBC aggregation increased in all Poly(hHb:HSA) formulations compared to acellular Hb, indicating that the presence of macromolecular species promotes cell–cell interactions at low shear. However, the magnitude of aggregation depended on the hHb:HSA mass ratio, with Poly(hHb₅₀:HSA₅₀) producing the highest RBC aggregation indices and PolyhHb₁₀₀ exhibiting the lowest. This

**Figure 16.** Measurement of colloid osmotic pressure in the solution of precursor hHb, T-state PolyhHb₁₀₀, and T-state Poly(hHb:HSA) conjugates.

suggests that HSA contributes to reversible intercellular interactions through depletion or bridging forces generated by large polymeric species.⁹¹ Despite the increase in aggregation, these interactions remained moderate and reversible, as reflected by the lack of stable rouleaux formation. Moderate, shear-dependent aggregation is physiologically beneficial, as it can reduce the apparent viscosity at low shear and promote plasma skimming in the microcirculation without compromising perfusion. Thus, the inclusion of HSA in the copolymer appears to balance aggregation dynamics, potentially enhancing microvascular flow properties while avoiding excessive RBC clustering.

All formulations demonstrated shear-thinning behavior, a characteristic feature of plasma and whole blood.^{91,92} The viscosity decreased with increasing shear rate and increased with protein concentration, with Poly(hHb₅₀:HSA₅₀) showing the strongest concentration-dependent response. In contrast, acellular Hb exhibited minimal concentration dependence and near-Newtonian behavior, indicating the absence of intermolecular interactions. The rheological scaling analysis further supported these trends, as all Poly(hHb:HSA) formulations displayed positive scaling exponents ($\alpha > 1$), indicating

Table 4. Concentration-dependent rheology parameters from paired flow sweeps (1000 → 10 → 1000 s⁻¹, 10 measurements per step, 37 °C; 2° one)^a

	precursor hHb	polyhHb ₁₀₀	poly(hHb ₇₅ :HSA ₂₅)	poly(hHb ₅₀ :HSA ₅₀)	poly(hHb ₂₅ :HSA ₇₅)
$n_{100}-n_{50}$ flow-behavior index (no units)	-0.092	0.102	0.031	0.135	0.095
K_{100}/K_{50} consistency ratio (no units)	2.06	0.849	1.55	1.37	0.857

^aValues are the flow-behavior index difference $\Delta n = n_{100} - n_{50}$ and the consistency ratio $K_{100}/K_{50} = 2^a$, obtained by fitting $\alpha(\gamma) = \ln(\eta_{100}/\eta_{50})/\ln(2) = a + s^* \ln(\gamma)$ over the quasi–power-law region. Positive Δn indicates the 100 mg/mL sample is less shear-thinning than the 50 mg/mL sample; $K_{100}/K_{50} > 1$ indicates a higher viscosity scale at 100 mg/mL. Concentrated co-polymer formulations are generally less shear-thinning than their dilute counterparts, with the strongest effect for Poly(hHb₅₀:HSA₅₀); hHb shows $\Delta n < 0$, indicating slightly greater shear-thinning at higher concentration.

Table 5. Colloid Osmotic Pressure (Units = mmHg)

concentration (mg/mL)	precursor hHb	polyhHb ₁₀₀	poly(hHb ₇₅ :HSA ₂₅)	poly(hHb ₅₀ :HSA ₅₀)	poly(hHb ₂₅ :HSA ₇₅)
10	4.28 ± 0.78 ^{a,b,c,d}	1.35 ± 0.47	1.78 ± 0.60	1.38 ± 0.66	2.30 ± 0.39
50	21.20 ± 0.85 ^{a,b,c,d}	2.20 ± 0.32	2.55 ± 0.41	4.80 ± 0.53 ^{b,c}	8.00 ± 0.75 ^{b,c,d}
100	38.75 ± 1.44 ^{a,b,c,d}	3.80 ± 0.26	5.38 ± 0.67	7.8 ± 0.99 ^b	11.75 ± 0.88 ^{b,c,d}

^avs Hb. ^bvs PolyhHb₁₀₀. ^cvs Poly(hHb₇₅:HSA₂₅). ^dvs Poly(hHb₅₀:HSA₅₀).

superlinear increases in viscosity with concentration. Poly(hHb₅₀:HSA₅₀) showed the highest α values and the largest positive slope in α versus $\ln \dot{\gamma}$, suggesting a stronger cohesive structure that reorganizes under shear but maintains fluidity. This adaptive rheology is advantageous for HBOC design because it allows the solution to behave like plasma, providing high viscosity and shear-thinning at low flow while maintaining low resistance at high shear rates, similar to native blood.

The COP revealed that HSA content directly influenced oncotic pressure. While acellular hHb exhibited the highest COP overall, its oncotic effect is largely nonphysiological due to its lack of macromolecular retention and rapid extravasation. Among the polymeric formulations, the COP increased with increasing HSA mass fractions. The significantly lower COP in PolyhHb₁₀₀ underscores the need for HSA incorporation to achieve vascular stability. Together, these findings show that integrating HSA into the copolymer structure not only improves rheological behavior but also enhances COP and plasma expansion properties essential for intravascular persistence.

4. CONCLUSION

This study presents a comprehensive biophysical and biochemical characterization of next-generation, HMW T-state Poly(hHb:HSA) conjugates synthesized at the bench scale. Analytical assessments using SEC-HPLC, DLS, and MALDI-TOF confirmed successful polymerization with minimal LMW species or unreacted hHb, minimizing potential risks of vascular leakage, NO scavenging, vasoconstriction, and hypertension. Among the formulations examined, T-state PolyhHb₁₀₀ and Poly(hHb₇₅:HSA₂₅) exhibited the highest yields (43% and 39%) and M_w ~2000 kDa, whereas HSA-enriched variants, including Poly(hHb₅₀:HSA₅₀) and Poly(hHb₂₅:HSA₇₅), produced lower yields (26% and 17%) and molecular weights (~875 and ~500 kDa). Despite this, the Poly(hHb₅₀:HSA₅₀) formulation demonstrated the most favorable biophysical and rheological behavior, establishing it as the optimal candidate for potential physiological applications.

Both Poly(hHb₇₅:HSA₂₅) and Poly(hHb₅₀:HSA₅₀) maintained metHb levels below the 10% safety threshold and within acceptable limits for HBOCs. Oxygen-binding analyses revealed an oxygen affinity (P_{50}) and oxygen offloading kinetics consistent with therapeutic ranges necessary for efficient tissue oxygenation under moderate oxygen tensions. Furthermore, both Poly(hHb₇₅:HSA₂₅) and Poly(hHb₅₀:HSA₅₀) demonstrated reduced haptoglobin binding, indicative of potentially slower clearance and prolonged intravascular retention. Both copolymers exhibited high viscosity, shear-thinning behavior, and reversible RBC aggregation, critical parameters for potentially maintaining microvascular flow, plasma volume, and vascular stability. Both copolymers exhibited comparable surface charge. Spectroscopic analysis of both copolymers confirmed preservation of the α -helical structure and an intact heme environment

following glutaraldehyde cross-linking. Oxidative stability and heme retention were maintained at similar levels for both copolymers, while catalase-like antioxidant activity remained sufficient to counter heme-mediated oxidative stress. Taken together, both copolymers offer the most balanced performance profile, combining moderate M_w and size, balanced protein composition, favorable rheology, oxidative stability, and oxygen transport characteristics. These characteristics position both copolymers as optimal next-generation HBOC candidates, providing a potentially promising platform for safe and effective blood substitute development.

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Author Contributions

Andre F. Palmer and Mohd A. Khan contributed to conceptual development. All authors contributed to experimental planning, data organization, and formal analysis of the study. Material synthesis was carried out by Mohd A. Khan. Mohd A. Khan, Tanmay Salvi and Griffin J. Beyer were responsible for data curation. Elliot Widd, Jacinda Martinez, Carlos Munoz and Pedro Cabrales performed all rheological studies, including

red blood cell aggregation and viscosity measurements, as well as COP measurements. The first version of the manuscript was written by Mohd A. Khan and Andre F. Palmer. Further revisions, editing, and preparation of the final manuscript for publication were completed by Mohd A. Khan, Tanmay Salvi, Griffin J. Beyer, Elliot Widd, Jacinda Martinez, Carlos Munoz, Pedro Cabrales and Andre F. Palmer. Funding acquisition was managed by Andre F. Palmer.

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