Functional Cross-Linked Hemoglobin Bis-tetramers: Geometry and Cooperativity†

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ABSTRACT: Hemoglobin-based oxygen carriers have been sought as stable, sterile alternatives to red cells in transfusions. Problems in clinical trials using cross-linked tetramers have led to proposals that larger assemblies of tetramers may alleviate some of the problems. A study of such assemblies requires materials with defined structures and physical properties. Evaluation of cross-linked bis-tetramers with inflexible linear links between the tetramers revealed that these have very low cooperativity in oxygen binding and would thus be inefficient as oxygen carriers. New, more flexible reagents were designed to cross-link and connect tetramers in two modes: with angular connectors that permit torsional movement (**1**−**3**) and with linear connectors that resemble previously studied systems (**4**−**6**). The resulting cross-linked bis-tetramers were produced in high yield and were isolated and characterized. Digest mapping showed that modifications were specifically introduced as expected at amino groups in the 2,3-bisphosphoglycerate binding sites within β subunits. Circular dichroism showed that the secondary structure of the globin chains is maintained while the microenvironment of the hemes is altered. The bis-tetramers derived from **1**−**3** have oxygen affinity ($P_{50} = 3.6-4.7$) and cooperativity ($n_{50} = 2.2-2.7$) that appear to be suitable for efficient oxygen delivery to hypoxic regions along with increased mass that is expected to minimize extravasation.

Cross-linked hemoglobins have received much attention for their potential use as an alternative oxygen carrier (hemoglobin-based oxygen carrier, "HBOC")[1] in transfusion medicine (*1, 2*). However, unforeseen difficulties related to safety (*3*) and efficacy have arisen in clinical trials (*4*−*6*), leading to a need for new materials with specific structural characteristics (*7*) that can be related to their performance. For example, acellular cross-linked hemoglobin tetramers cause serious problems associated with vasoactivity (*8, 9*). Larger species, consisting of heterogeneous oligomers (*10, 11*) or conjugates of polyethylene glycol (*12*), have fewer problems associated with vasoactivity (*13*). Heterogeneous oligomers (*14, 15*) are problematic in that the composition and structure of the materials being administered cannot be precisely determined and thus cannot be correlated with clinical events (*6, 7*). For materials with PEG chains (*16*), a major problem is that the additional mass comes without additional oxygen-carrying capacity, requiring administration of large amounts of material.

As an alternative approach to overcome the problems with the above materials, we have been developing reagents that react selectively within hemoglobin tetramers and which also create cross-links between tetramers (*17*−*19*). These structurally defined entities are in principle equal to cross-linked hemoglobin in oxygen-carrying capacity, thus overcoming the known problems associated with procedures that are nonspecific (*14, 15*) or that add mass without increased oxygenation capacity (*16*). Previously, in designing reagents to link tetramers, we extended the basic structural design principles that had been successful in creating links efficiently between subunits within tetramers: (1) anionic acylation reagents direct reaction to amino groups in the highly cationic region within hemoglobin where the affector 2,3-bisphosphoglycerate (BPG) binds, (2) the reacting sites are derived from isophthalic acid, an arrangement known to give efficient cross-linking, and (3) connecting linkages are from arrays containing exclusively sp^2 hybridized centers in order to maintain rigidity in the reagents, producing defined spans and preventing internal association of the reagent through folding (*20*−*24*). These materials showed the desired reaction efficiency and specificity in creating arrays of cross-linked hemoglobin of defined structure.

Our examination of the oxygenation properties of those assemblies revealed that they are associated with oxygen-binding curves with very low Hill coefficients (*20*−*24*) and thus lack cooperativity in oxygen binding and release. Examination of oxygenation curves in terms of circulatory delivery clearly reveals the practical necessity of a having high degree of cooperativity in an effective oxygen transport agent (*25*). As a first step in designing reagents that will produce assemblies of cross-linked hemoglobin that would exhibit cooperative oxygen transfer, we examined the effect of introducing a single structural perturbation, producing

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[1] Abbreviations: HBOC, hemoglobin-based oxygen carrier; Hb, hemoglobin; THF, tetrahydrofuran; DCM, dichloromethane; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; DMSO, dimethyl sulfoxide; CD, circular dichroism; MALDI-TOF, matrix-assisted laser desorption ionization time of flight.

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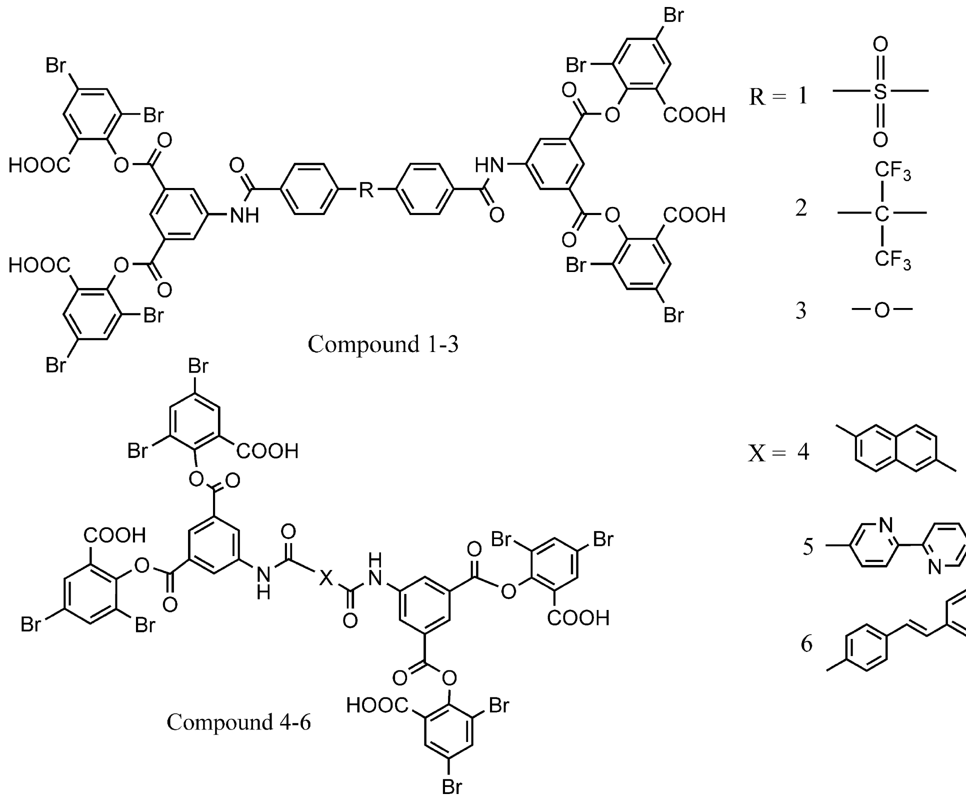
Chart 1



linkages with a single tetrahedral center at the midpoint of the intertetramer linkage, with the rest remaining planar. This would result in torsional flexibility at the central point in the linkages between tetramers. We have now produced the reagents (**1**−**3**), examined their reactions with hemoglobin, and assessed the structures and properties of the resulting protein assemblies. We find that the reagents are efficient and the resulting assemblies have the desired increase in cooperativity associated with effective oxygen delivery. In order to further test the validity of these results, we prepared very similar materials with planar linear linkages (**4**−**6**) for comparison (see Chart 1). As predicted, these linkers produce cross-linked connected hemoglobins with low cooperativity in their oxygen binding.

EXPERIMENTAL PROCEDURES

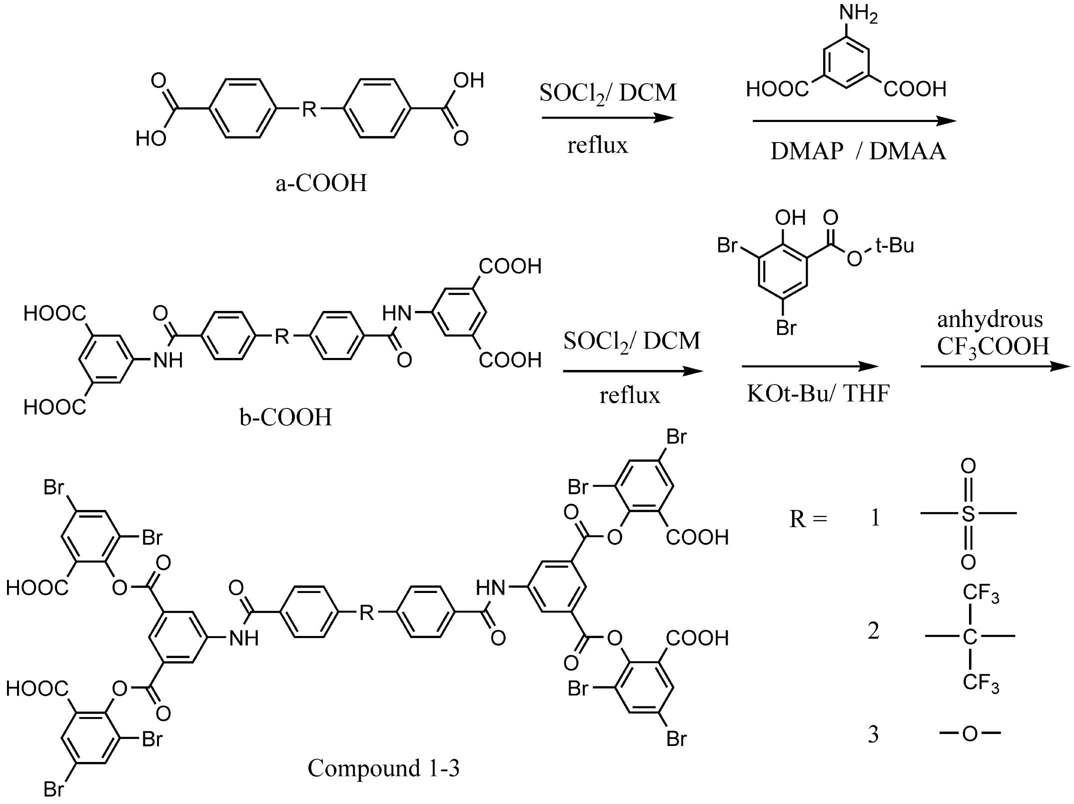
Solvents. THF was dried by distillation from metallic sodium. Acetone was dried by distilling from dry gypsum. Other materials and reagents were used as purchased.

Hemoglobin. The protein was purified from human red cells by the method described by Winslow et al. (*26*) and was stored in doubly distilled water at 0−5 °C. Hemoglobin stored as the carbonmonoxy form is stable for extended periods under these conditions. Each sample was checked for oxidation prior to use. Concentrations of hemoglobin solutions were determined using the cyanomethemoglobin assay (*27*). The purity of hemoglobin was determined through the reverse-phase HPLC analysis described by Jones (*28*).

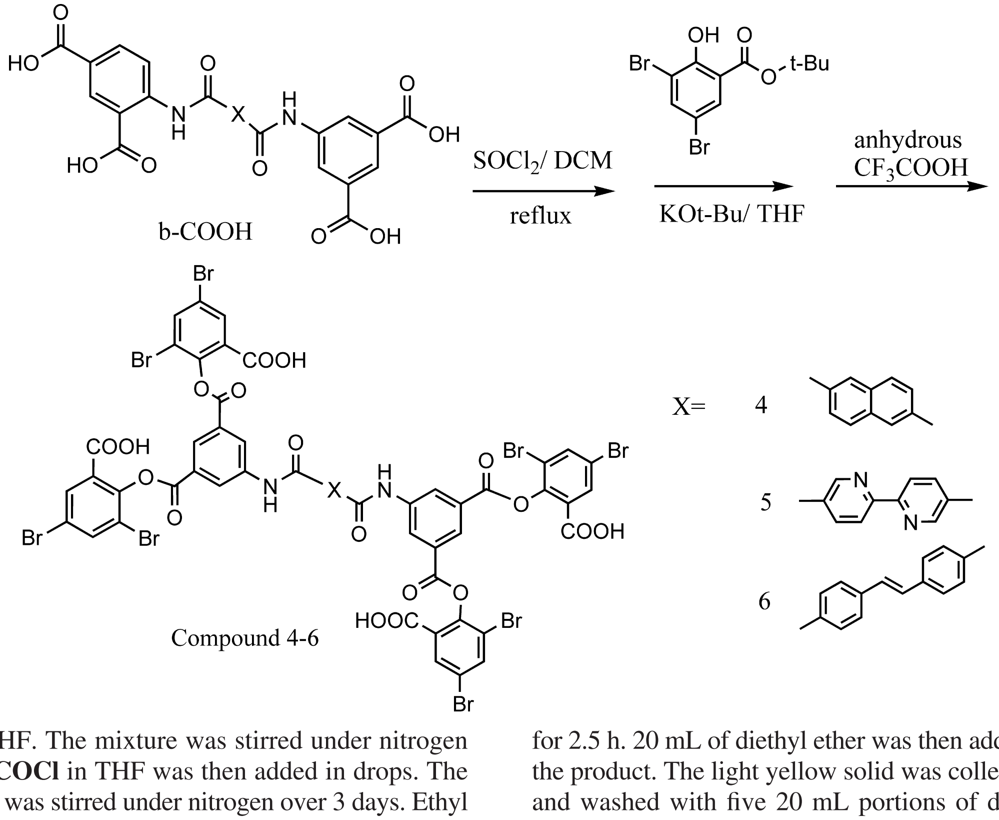
*5,5′-[Sulfonyl bis(1,4-phenylenecarbonylimino)]bis-1,3-benzenedicarboxylic Acid (***1b-COOH***).* 4,4′-Dicarboxydiphenyl sulfone (**1a-COOH**, 2 mmol, 0.62 g) was dissolved in 15 mL of dichloromethane containing 5 mL of thionyl chloride. The clear yellow solution was refluxed under nitrogen for 24 h. The solvent was evaporated to generate **1a-COCl** as a light yellow powder. 5-Aminoisophthalic acid (0.82 g, 4.5 mmol) and 4-(dimethylamino)pyridine (0.055 g, 0.45 mmol) were dissolved in 5 mL of anhydrous *N*,*N*-dimethylacetamide under nitrogen. **1a-COCl** was dissolved in 5 mL of anhydrous *N*,*N*-dimethylacetamide and added in drops. The mixture was stirred under nitrogen for 96 h to give a light yellow solution. The reaction mixture was then transferred to a 250 mL flask. 150 mL of 5% HCl was added to precipitate the product and dissolve excess 5-aminoisophthalic acid and 4-(dimethylamino)pyridine. The solid was washed by another 150 mL of 5% HCl followed by 100 mL × 4 of water and isolated by centrifugation. The damp product was freeze-dried and heated overnight to give a pale yellow powder (**1b-COOH**, 1.1 g, 83% yield). ^{1}H NMR (300 Hz, DMSO-d_6): δ ppm 13.30 (broad peak, COOH), 10.81 (s, 2H, CONH), 8.64 (d, $^4J = 1.5$ Hz, 4H, ArH), 8.23 (t, $^4J = 1.5$ Hz, 2H, ArH), 8.22 (s, 8H, ArH). ^{13}C NMR (300 Hz, DMSO-d_6): δ (10 peaks) 166.42, 164.51, 143.14, 139.45, 139.26, 131.73, 129.26, 127.82, 125.36, 124.73. High-resolution ESI-MS (DMSO): 631.0633 ($[M - H]^-$ $C_{30}H_{19}N_2O_{12}S$, 631.0664 calculated, −4.9 ppm difference). Melting point: 245−246 °C.

*N,N′-Bis[bis(3,5-dibromosalicyl)isophthalyl]-5,5′-[sulfonyl bis(1,4-phenylenecarbonylimino)]bis-1,3-benzenedicarboxylate (***1***,* **DBSS***).* 0.126 g (0.2 mmol) of **1b-COOH** was dissolved in 15 mL of dichloromethane containing 5 mL of thionyl chloride. The light yellow solution was refluxed under nitrogen for 18 h. Thionyl chloride was evaporated under vacuum, and the pale yellow solid was pumped for 2 h. The resulting **1b-COCl** was then dissolved in 5 mL of freshly dried THF, giving a yellow solution. *tert*-Butyl 3,5-dibromosalicylate (*29*), 0.28 g (0.8 mmol), and 0.090 g (0.8 mmol) of potassium *tert*-butoxide were combined and dissolved in

Scheme 1: Synthesis of Tetrakis-dibromosalicylates (**1**−**3**) from Dicarboxylic Acids



Scheme 2: Synthesis of Tetrakis-dibromosalicylates **4**−**6** from Tetrakis-carboxylic Acids



15 mL of dry THF. The mixture was stirred under nitrogen for 40 min. **1b-COCl** in THF was then added in drops. The reaction mixture was stirred under nitrogen over 3 days. Ethyl acetate (30 mL) was added to extract the product. The organic layer was washed with five 20 mL portions of water and dried with calcium chloride tablets. After filtration, ethyl acetate was evaporated, leaving a pale yellow solid. Anhydrous trifluoroacetic acid (TFA), 15 mL, was added to dissolve the solid, and the solution was stirred under nitrogen for 2.5 h. 20 mL of diethyl ether was then added to precipitate the product. The light yellow solid was collected by filtration and washed with five 20 mL portions of diethyl ether and dried under vacuum (**1**, **DBSSPB**, 0.27 g, 78% yield). ^{1}H NMR (300 Hz, DMSO-d_6): δ ppm 13.37 (broad peak, COOH), 11.11 (s, 2H, CONH), 9.01 (d, 4H, $^4J = 1.5$ Hz, ArH), 8.52 (t, 2H, $^4J = 1.5$ Hz, ArH), 8.36 (d, 4H, $^4J = 2.4$ Hz, ArH), 8.23, 8.24 (m, 8H, ArH), 8.11 (d, 4H, $^4J = 2.4$ Hz, ArH). ^{13}C NMR (300 Hz, DMSO-d_6): δ ppm (17 peaks)

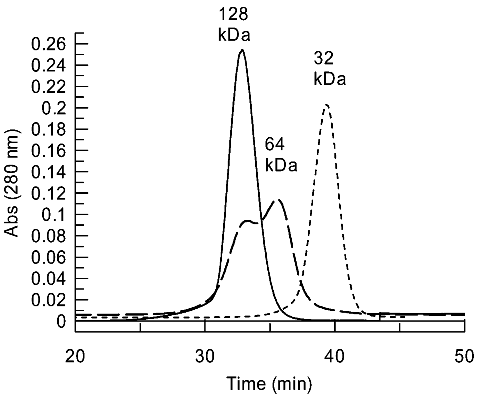


FIGURE 1: Sephadex G-200 size-exclusion chromatography analysis of hemoglobin species under conditions that cause dissociation of tetramers into dimers: native hemoglobin (dotted line); cross-linked bis-tetramer (solid line); cross-linked hemoglobin mixture from reaction with **1** (dashed line).

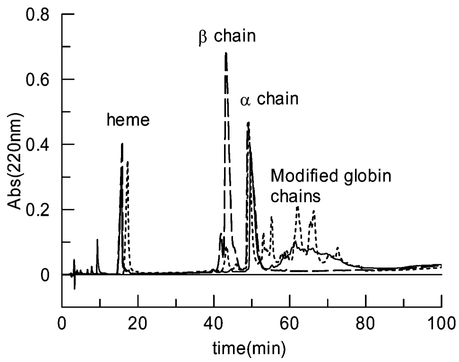


FIGURE 2: C4 reverse-phase HPLC analysis of hemoglobin globin chains: native protein (dashed line); modified hemoglobin mixture (dotted line); cross-linked hemoglobin bis-tetramer (solid line).

Table 1: Product Composition (%) in Reactions between Deoxyhemoglobin and Different Reagents

reagents	cross-linked bis-tetramer (128 kDa)	cross-linked tetramer (64 kDa)	unmodified
1	42	55	<5
2	27	70	<5
3	37	60	<5
4	31	39	30
5	40	55	<5
6	26	45	29

164.75, 163.42, 162.34, 157.211, 148.61, 146.52, 143.29, 129.56, 129.35, 127.86. 127.67, 126.20, 119.47, 119.26, 104.29, 103.30. High-resolution ESI-MS (DMSO): 870.7022 ($[M - 2H]^{2-}$, $C_{58}H_{26}N_2O_{20}S^{79}Br_4{}^{81}Br_4$, 870.7097 calculated, −8.7 ppm difference). Melting point: >300 °C.

*2,2′-[Hexafluoropropanyl bis(1,4-phenylenecarbonylimino)]bis-1,3-benzenedicarboxylic Acid (**2b-COOH**).* 2,2-Bis(4-carboxyphenyl) (**2a-COOH**, 3 mmol, 1.2 g) was dissolved in 10 mL of dichloromethane containing 15 mL of thionyl chloride. The clear yellow solution was refluxed under nitrogen for 24 h. The solvent was evaporated to generate **2a-COCl** as a light yellow oil. 5-Aminoisophthalic acid (1.22 g, 6.75 mmol) and 4-(dimethylamino)pyridine (0.082 g, 0.67 mmol) were dissolved in 10 mL of anhydrous *N*,*N*-dimethylacetamide under nitrogen. **2a-COCl** was dissolved in 5 mL of anhydrous *N*,*N*-dimethylacetamide and added in drops. The mixture was stirred under nitrogen for 96 h to give a brown solution. 150 mL of 5% HCl was added to precipitate the product and dissolve excess 5-aminoisophthalic acid and 4-(dimethylamino)pyridine. The solid was further washed with 150 mL of 5% HCl followed by four portions of 150 mL of water and isolated by centrifugation. The material was freeze-dried and then heated overnight to give a white powder (**2b-COOH**, 1.8 g, 84% yield). ^1H NMR (300 Hz, DMSO-d_6): δ 13.28 (broad peak, COOH), 10.78 (s, 2H, CONH), 8.67 (d, $^4J = 1.5$ Hz, 4H, ArH), 8.24 (t, $^4J = 1.5$ Hz, 2H, ArH), 8.10 (d, 4H, $^3J = 8.4$ Hz, ArH), 7.57 (d, 4H, $^3J = 8.4$ Hz, ArH). ^{13}C NMR (300 Hz, DMSO-d_6): δ ppm (10 peaks) 166.50, 165.08, 139.68, 135.72, 135.24, 131.74, 129.90, 128.30, 125.23, 124.74. High-resolution ESI-MS (DMSO): 717.0920 ($[M - H]^-$ $C_{33}H_{19}N_2O_{10}F_6$, 717.0949 calculated, −4.0 ppm difference). Melting point: 242−244 °C.

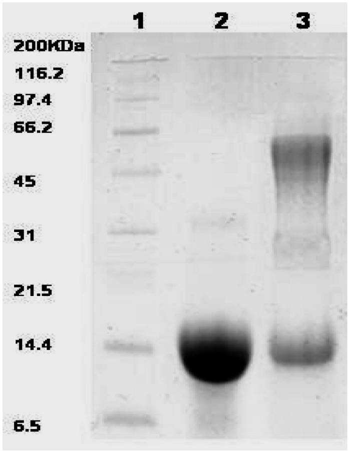


FIGURE 3: SDS−PAGE analysis: 1, mass standards; 2, native hemoglobin; 3, cross-linked hemoglobin bis-tetramers.

*N,N′-Bis[bis(3,5-dibromosalicyl)isophthalyl]-2,2′-[hexafluoropropanyl bis(1,4-phenylenecarbonylimino)]bis-1,3-benzenedicarboxylate (**2**, **DBSH**).* 0.214 g (0.3 mmol) of **2b-COOH** was activated as tetracarbonyl chloride and reacted with 0.423 g (1.2 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.135 g (1.2 mmol) of potassium *tert*-butoxide. After extraction and deprotection, the product was isolated as a yellow powder (**2**, DBSH, 0.32 g, 58% yield). ^1H NMR (300 Hz, DMSO-d_6): δ ~13.2 (broad peak, COOH), 11.04 (s, 2H, CONH), 9.03 (d, 4H, $^4J = 1.5$ Hz, ArH), 8.52 (t, 2H, $^4J = 1.5$ Hz, ArH), 8.36 (d, 4H, $^4J = 2.4$ Hz, ArH), 8.16 (d, 4H, $^3J = 8.4$ Hz, ArH), 8.12 (d, 4H, $^4J = 2.4$ Hz, ArH), 7.59 (d, 4H, $^3J = 8.4$ Hz, ArH). ^{13}C NMR (300 Hz, DMSO-d_6): δ (15 peaks) 166.44, 163.44, 162.38, 152.76, 138.99, 131.69, 131.03, 129.88, 128.97, 128.26, 126.10, 119.47, 115.52, 104.29, 103.41. High-resolution ESI-MS (DMSO): 913.7166 ($[M - 2H]^{2-}$ $C_{61}H_{26}N_2O_{18}F_6{}^{79}Br_4{}^{81}Br_4$, 913.7240 calculated). Melting point: >300 °C.

*5,5′-[Oxy bis(1,4-phenylenecarbonylimino)]bis-1,3-benzenedicarboxylic Acid (**3b-COOH**).* 4,4′-Dicarboxydiphenyl ether (**3a-COOH**, 3 mmol, 0.78 g) was dissolved in 10 mL of dichloromethane and 15 mL of thionyl chloride. The light yellow solution was refluxed under nitrogen for 24 h. The solvent was evaporated, leaving **3a-COCl** as a light yellow sticky solid. 5-Aminoisophthalic acid (1.3 g, 7 mmol) and 4-(dimethylamino)pyridine (0.085 g, 0.7 mmol) were dissolved in 10 mL of anhydrous *N*,*N*-dimethylacetamide under N_2. **3a-COCl** was dissolved with 5 mL of anhydrous *N*,*N*-dimethylacetamide and added in drops. The mixture was stirred under nitrogen for 96 h to give a light yellow solution.

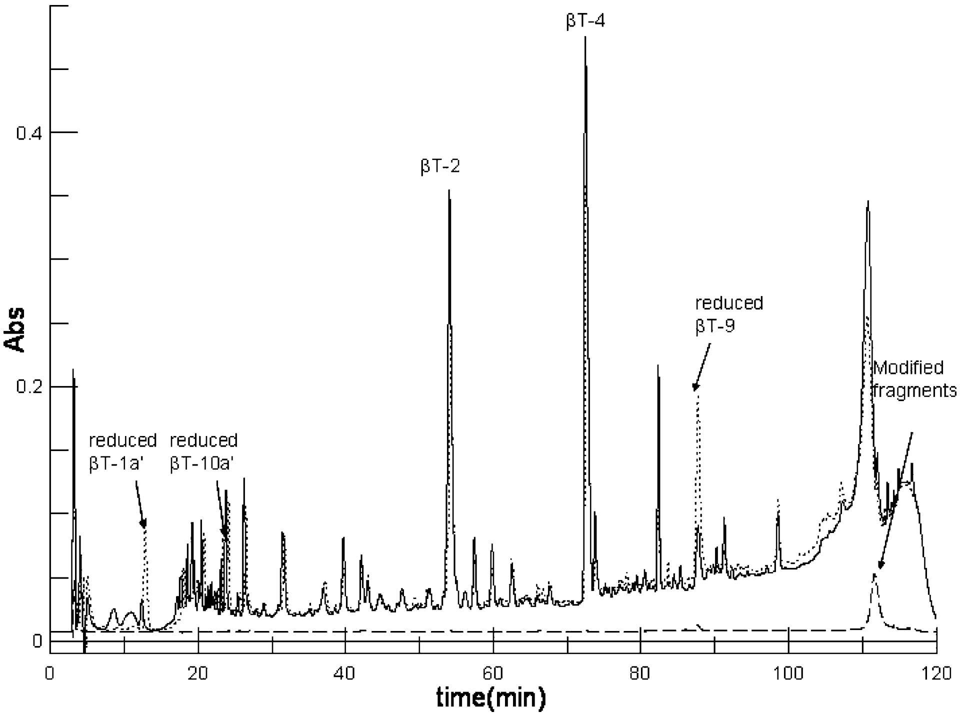


FIGURE 4: Trypsin−Glu-C digestion fragment analysis by HPLC on a C18 reverse-phase column: native β chains (dotted line, monitored at 220 nm), modified β chains (solid line, monitored at 220 nm), and modified β chains (dashed line, monitored at 258 nm, where isophthalates absorb).

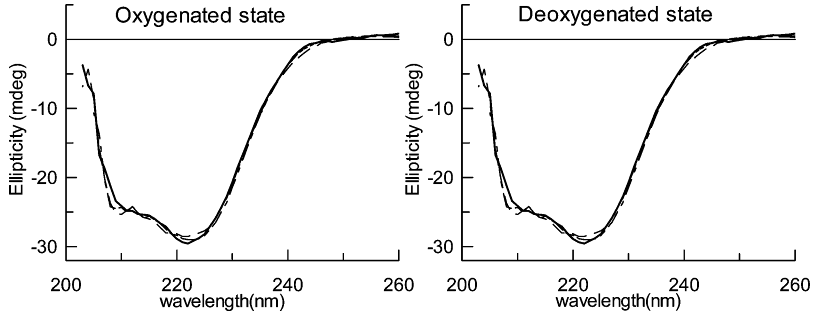


FIGURE 5: CD spectra (200−260 nm) of native hemoglobin (solid line) and bis-tetramer **1** (dashed line), bis-tetramer **2** (dotted line), and bis-tetramer **3** (dashed-dotted line).

150 mL of 5% HCl was added to precipitate the product and dissolve the excess 5-aminoisophthalic acid and 4-(dimethylamino)pyridine. The solid was washed by another 150 mL of 5% HCl followed by 150 mL × 4 of water and isolated by ultracentrifugation. The damp product was freeze-dried and kept in the oven overnight to give a light yellow powder (**3b-COOH**, 1.7 g, 96% yield). ^{1}H NMR (400 Hz, DMSO-d_6): δ ppm 13.3 (broad peak, COOH), 10.58 (s, 2H, CONH), 8.68 (d, $^4J = 1.6$ Hz, 4H, ArH), 8.22 (t, $^4J = 1.6$ Hz, 2H, ArH), 8.11 (d, 4H, $^3J = 8.8$ Hz, ArH), 7.24 (d, 4H, $^3J = 8.8$ Hz, ArH). ^{13}C NMR (300 Hz, DMSO-d_6): δ (10 peaks) 166.58, 164.94, 158.91, 139.89, 131.72, 130.27, 129.80, 124.98, 124.73, 118.59. High-resolution ESI-MS (DMSO): 583.0972 ($[M - H]^-$ $C_{30}H_{19}N_2O_{11}$, 583.0994 calculated, −3.8 ppm difference). Melting point: 234−235 °C.

*N,N′-Bis[bis(3,5-dibromosalicyl)isophthalyl]-5,5′-[oxybis(1,4-phenylenecarbonylimino)]bis-1,3-benzenedicarboxylic Acid (*3*,* **DBSO***).* 0.29 g (0.5 mmol) of **3b-COOH** was activated as the tetracarbonyl chloride with thionyl chloride and then reacted with 0.71 g (2 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.23 g (2 mmol) of potassium *tert*-butoxide. After extraction and deprotection as described previously, the product was isolated as a pale yellow solid (**3**, **DBSO**, 0.58 g, 69% yield). ^{1}H NMR (300 Hz, DMSO-d_6): δ 13.44 (broad peak, COOH), 10.86 (s, 2H, CONH), 9.05 (d, 4H, $^4J = 1.2$ Hz, ArH), 8.51 (t, 2H, $^4J = 1.2$ Hz, ArH), 8.37 (d, 4H, $^4J = 2.4$ Hz, ArH), 8.16 (d, 4H, $^3J = 8.7$ Hz, ArH), 8.12 (d, 4H, $^4J = 2.4$ Hz, ArH), 7.29 (d, 4H, $^3J = 8.8$ Hz, ArH). ^{13}C NMR (300 Hz, DMSO-d_6): δ (16 peaks) 165.20, 163.46, 162.45, 159.05, 146.57, 140.98, 138.99, 133.49, 130.38, 129.50, 129.44, 127.67, 126.12, 119.47, 119.30, 118.66. High-resolution ESI-MS (DMSO): 846.7230 ($[M - 2H]^{2-}$ $C_{58}H_{26}N_2O_{19}{}^{79}Br_4{}^{81}Br_4$, 846.7262 calculated, −3.8 ppm difference). Melting point: >300 °C.

*N,N′-Bis[bis(3,5-dibromosalicyl)isophthalyl]-2,6-naphthalenedicarboxylate (*4*,* **DBSN***).* 0.11 g (0.2 mmol) of *N,N′*-bis(isophthalyl)-2,6-naphthalenedicarboxylate (*23*) (**4b-COOH**) was activated as the tetracarbonyl chloride with 15 mL of thionyl chloride and then reacted with 0.28 g (0.8 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.090 g (0.8 mmol) of potassium *tert*-butoxide in 15 mL of dry THF for 2 days. After extraction and TFA cleavage of the *tert*-butyl ester, the product was isolated as a pale yellow solid (**4**,

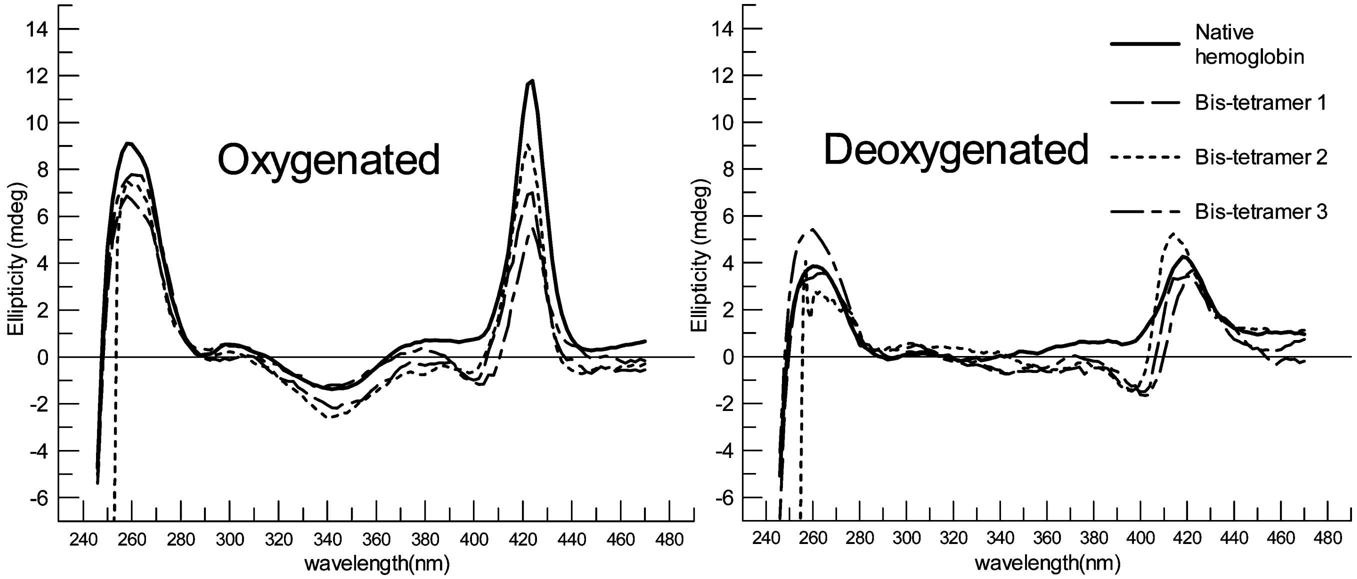


FIGURE 6: CD spectra (240−470 nm) of native hemoglobin (solid line) and bis-tetramer **1** (dashed line), bis-tetramer **2** (dotted line), and bis-tetramer **3** (dashed-dotted line) (optimized).

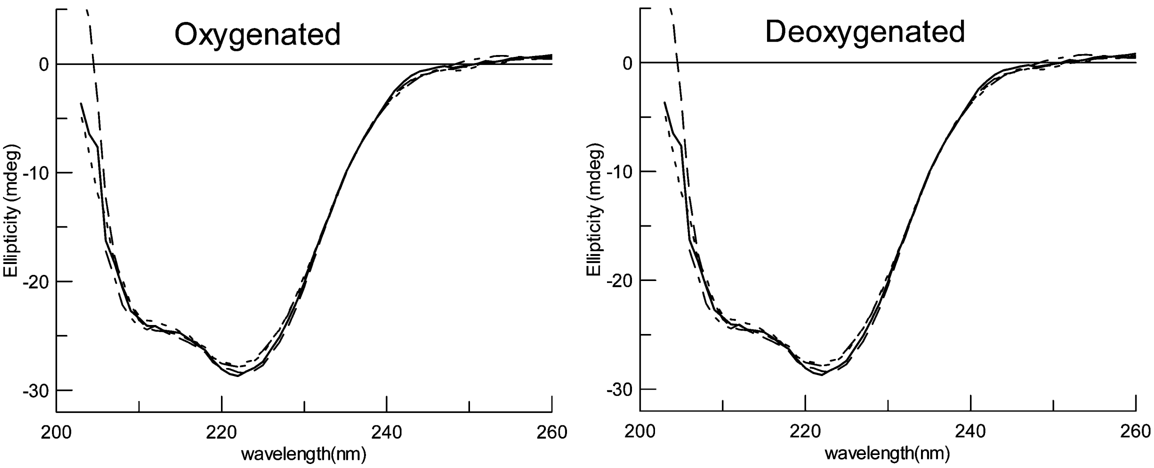


FIGURE 7: CD spectra (200−260 nm) of native hemoglobin (solid line) and bis-tetramer **4** (dashed line), bis-tetramer **5** (dotted line), and bis-tetramer **6** (dashed-dotted line).

DBSN, 0.21 g, 64% yield). ^{1}H NMR (DMSO-d_6): δ 11.12 (2H, s, NH), 9.09 (4H, d, ^4J = 1.5 Hz ArH), 8.74 (2H, dd, ^3J = 7.5 Hz, ArH), 8.52 (2H, t, ^4J = 1.5 Hz, ArH), 8.36 (4H, d, ^4J = 2.1 Hz, ArH), 8.23 (2H, d, ^4J = 1.5 Hz ArH), 8.11 (4H, d, ^4J = 2.7 Hz, ArH), 8.17 (2H, d, ^3J = 8.4 Hz, ArH). High-resolution ESI-MS (DMSO): 822.7235 ([M − 2H]$^{2-}$ $C_{56}H_{24}N_2O_{18}{}^{79}Br_7{}^{81}Br$, 822.7240 calculated, −0.7 ppm difference). Melting point: >300 °C.

*N,N′-Bis[bis(3,5-dibromosalicyl)isophthalyl]-2,2′-bipyridinyl-5,5′-dicarboxylate (***5**, **DBSBP***).* 0.077 g (0.14 mmol) of *N,N′*-bis(isophthalyl)-2,2′-bipyridinyl-5,5′-dicarboxylate (*24*) (**5b-COOH**) was converted to tetracarbonyl chloride (an orange solid) and reacted with 0.20 g (0.56 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.061 g (0.56 mmol) of potassium *tert*-butoxide in THF for 2 days. After extraction and cleavage of the *tert*-butyl ester with TFA, the product was isolated as a yellow solid (**5**, **DBSBP**, 0.11 g, 31% yield). ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.14 (2H, s, NH), 9.33 (2H, s, PyrH), 9.05 (4H, d, ^4J = 1.5 Hz, ArH), 8.66 (2H, d, ^3J = 7.8 Hz, PyrH), 8.61 (2H, d, ^3J = 7.8 Hz, PyrH), 8.52 (2H, t, ^4J = 1.5 Hz, ArH), 8.36 (4H, d, ^4J = 2.4 Hz, ArH), 8.11 (4H, d, ^4J = 2.4 Hz, ArH). High-resolution ESI-MS (DMSO): 839.7240 ([M − 2H]$^{2-}$, 839.7275 calculated for $C_{56}H_{24}N_4O_{18}{}^{79}Br_4{}^{81}Br_4$, 4.0 ppm difference). Melting point: 253−254 °C.

*N,N′-Bis[bis(3,5-dibromosalicyl)isophthalyl]-trans-stilbene-4,4′-dicarboxylate (***6**, **DBSS***).* 0.12 g (0.2 mmol) of *N,N′*-bis(isophthalyl)-*trans*-stilbene-4,4′-dicarboxylate (*24*) (**6b-COOH**) was transformed to tetracarbonyl chloride as a yellow solid. After reacting with 0.28 g (0.8 mmol) of *tert*-butyl 3,5-dibromosalicylate and 0.090 g (0.8 mmol) of potassium *tert*-butoxide for 2 days followed by the deprotection in anhydrous TFA, a yellow powder was isolated (**6**, **DBSS**, 0.26 g, 76% yield). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.89 (2H, s, NH), 9.90 (4H, d, ^4J = 1.5 Hz, ArH), 8.49 (2H, t, ^4J = 1.5 Hz, ArH), 8.35 (4H, d, ^4J = 2.4 Hz, ArH), 8.11 (4H, d, ^4J = 2.7 Hz, ArH), 8.08 (4H, d, ^3J = 8.7 Hz, ArH), 7.84 (4H, d, ^3J = 8.7 Hz, ArH), 7.54 (2H, =CH). High-resolution ESI-MS (DMSO): 851.7351 ([M − 2H]$^{2-}$, 851.7366 calculated for $C_{60}H_{28}N_2O_{18}{}^{79}Br_4{}^{81}Br_4$, −1.8 ppm difference). Melting point: >300 °C.

Reaction with Hemoglobin. Native carbonmonoxyhemoglobin (HbCO) was transferred to sodium borate buffer (0.05 M, pH 9.0) through a Sephadex G-25 gel-filtration column. The concentration of hemoglobin tetramers was maintained at about 0.6 mmol/L in the reaction. HbCO was converted

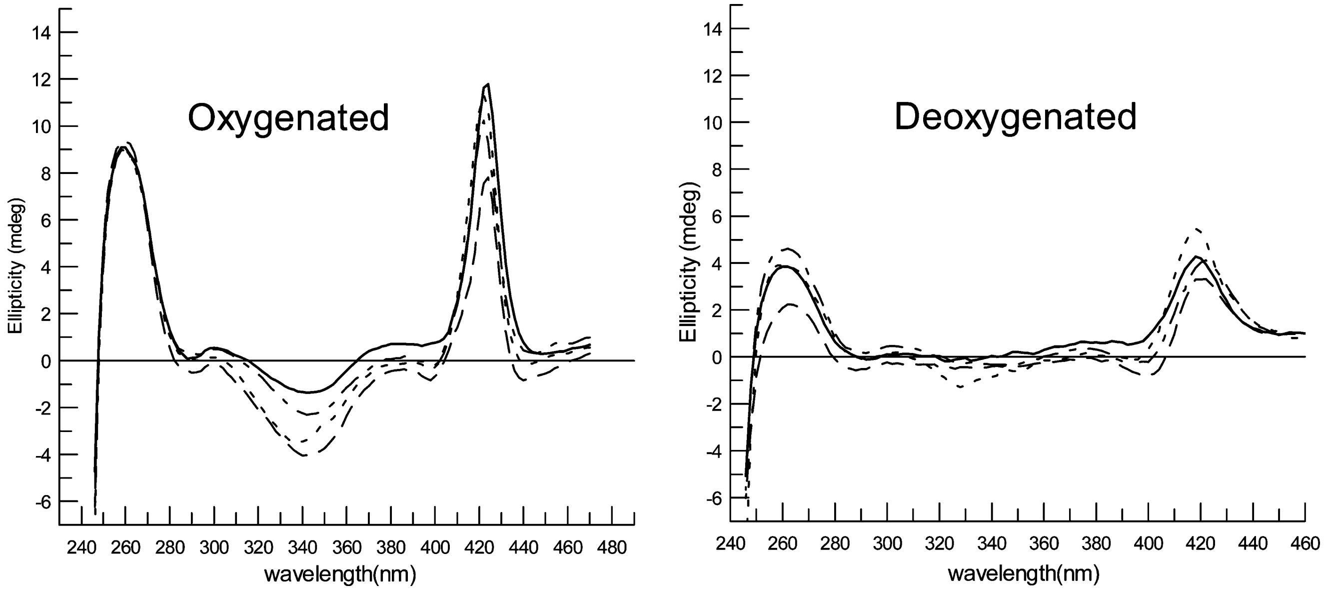


FIGURE 8: CD spectra (240−470 nm) of native hemoglobin (solid line) and bis-tetramer **4** (dashed line), bis-tetramer **5** (dotted line), and bis-tetramer **6** (dashed-dotted line).

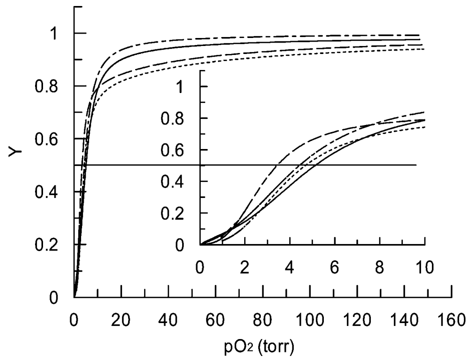


FIGURE 9: Oxygen-binding curves of modified hemoglobin species: native hemoglobin (solid line), bis-tetramer **1** (dashed line), bis-tetramer **2** (dashed-dotted line), and bis-tetramer **3** (dotted line).

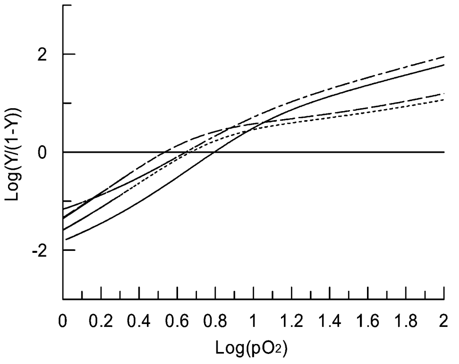


FIGURE 10: Hill plots of modified hemoglobin species: native hemoglobin (solid line), bis-tetramer **1** (dashed line), bis-tetramer **2** (dashed-dotted line), and bis-tetramer **3** (dotted line).

to oxygenated hemoglobin (oxyHb) and deoxygenated hemoglobin (deoxyHb) (*23*). Separate solutions containing 2 equiv of each reagent (**1**−**6**) were prepared using DMSO (∼50 μL) and injected into 10 mL of solutions of hemoglobin under nitrogen. The reaction was carried out at 37 °C under nitrogen overnight and flushed with carbon monoxide for 20 min. The reaction mixture was filtered and washed through a Sephadex G-25 gel-filtration column into MOPS buffer (0.1 M, pH 7.2) to remove reagents and organic solvent. The products were stored at 4 °C in sealed vials.

SDS−PAGE Analysis. The molecular weights of constituent proteins were estimated using polyacrylamide gel (12%, Tris-HCl) electrophoresis. Discontinuous Tris-HCl polyacrylamide gels were comprised of 12% separating gel (pH 8.8) and 5% stacking gel (pH 6.8) both with 10% sodium dodecyl sulfate. Protein samples were treated with 2-mercaptoethanol and sodium dodecyl sulfate (*30*). Globin chains were further denatured by heating at 100 °C for 15 min before loading on the gel. Finished gels were stained with Coomassie Brilliant Blue (*20*).

Chromatographic Analysis of Modified Hemoglobins. Globin analysis and separation by reverse-phase HPLC with a C-4 column (*31*) were applied using gradients of acetonitrile (20−60%) and 0.1% trifluoroacetic acid as the developer (*32*) with a 330 Å pore Vydac C-4 250 × 4.6 mm column for analysis and 250 × 12 mm column for preparative experiments. The effluent was monitored at 220 nm.

Size-exclusion chromotography on a Sephadex G-200 HR column (300 × 10 mm) under partially dissociating conditions (37.5 mM Tris-HCl, pH 7.4, 0.75 M magnesium chloride) provided the molecular size distribution of modified hemoglobins. The effluent was monitored at 280 nm. The integrated areas of peaks were used to estimate the molecular size distributions of reaction mixtures.

Isolation of Modified Hemoglobins. The modified hemoglobins were separated by gel-filtration chromatography (Sephadex G-100, 1000 × 35 mm) eluting with 25 mM Tris-HCl, pH 7.4, containing 0.75 M magnesium chloride. Fractions were concentrated and analyzed using Superdex G-200 size-exclusion chromotography, C4 reverse-phase analytical HPLC, and SDS−PAGE.

Tryptic and Glu-C Digest. Native hemoglobin β globin chains and cross-linked hemoglobin β globin chains were collected by preparative C4 reverse-phase HPLC and lyophilized. The β globin chains were then dissolved with 8 M urea in 80 mM ammonium bicarbonate (pH 8.5) and kept at room temperature for 4 h. The solution was diluted to 2 M urea with 80 mM ammonium bicarbonate (pH 8.5) and then hydrolyzed by TPCK-treated trypsin (0.1 mg/mL, 2% by weight of globin chain). The tryptic digestion solution

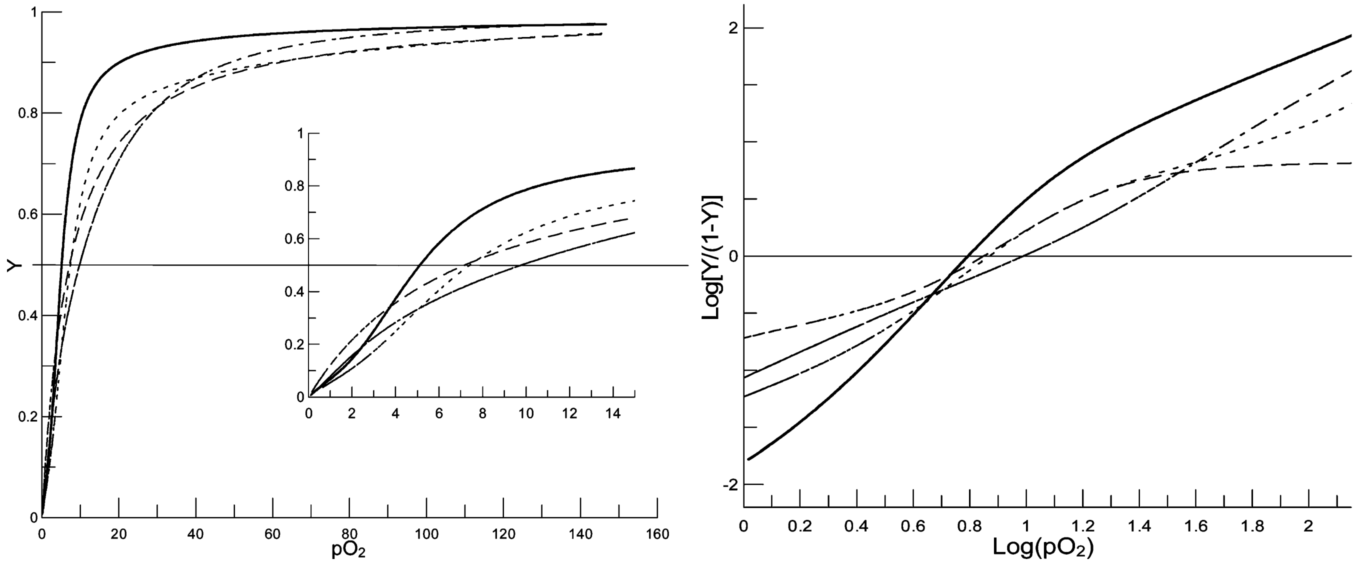


FIGURE 11: Oxygen-binding curves and Hill plot of modified hemoglobin species: native hemoglobin (solid line), bis-tetramer **4** (dashed line), bis-tetramer **5** (dotted line), and bis-tetramer **6** (dashed-dotted line).

Table 2: P_{50} and n_{50} Values of Native and Six Cross-Linked Hemoglobin Bis-tetramers

sample	P_{50} (±0.1 torr)	n_{50} ± 0.1
native hemoglobin	5.1	2.7
bis-tetramer **1**	3.6	2.7
bis-tetramer **2**	4.4	2.2
bis-tetramer **3**	4.7	2.5
bis-tetramer **4**	7.0	1.5
bis-tetramer **5**	7.4	1.8
bis-tetramer **6**	9.8	1.6

contained 1 mg/mL globin and 0.02 mg/mL trypsin in 80 mM ammonium bicarbonate (pH 8.5) and 2 M urea. The solution was kept at 37 °C for 24 h and heated at 100 °C for 5 min to inactivate trypsin. The solution was then diluted to 1 M urea with 80 mM ammonium bicarbonate buffer (pH 8.5) and further digested by endoproteinase Glu-C *Staphylococcus aureus* V8 (1 mg/mL, 1% of mass of total protein). The mixture, which contained 0.5 mg/mL globin chains and 0.005 mg/mL Glu-C protease, was kept at 37 °C for 72 h and heated at 100 °C for 5 min. The hydrolysates were lyophilized. An aliquot (~300 μg) of each sample was dissolved in 50 μL of water and analyzed by reverse-phase HPLC using a C18 column (250 × 4.5 mm). Peptide fragments were separated using gradients of acetonitrile from 0% to 100% with 0.1% THF as the developer (*32*). The peaks for peptide fragments were labeled based upon the MALDI-TOF MASS analysis results of hydrolysates and reference data (*33, 34*). The effluents were monitored at 220 nm for all peptide fragments and 258 nm for phthalates (*34*).

Oxygen-Binding Analysis. Purified cross-linked hemoglobin (~1 g/L) and native hemoglobin (~1 g/L) were transferred to sodium phosphate buffer (I = 0.1 M, pH = 7.4) and oxygenated. Oxygen-binding curves were obtained using a Hemox Analyzer at 25 °C. This temperature was used in order to compare our results with previously work. The data were fit to the Adair equation to obtain P_{50} and n_{50} (*35*).

Circular Dichroism Spectroscopy Conformational Study.

Native hemoglobin and cross-linked hemoglobin in carbon monoxy and oxy forms were studied by CD at both short (200−260 nm) and long (260−470 nm) wavelengths at room temperature (*36*). Hemoglobin samples were prepared as 10 mL solutions of the same globin chain (heme) concentration in the sodium phosphate buffer (I = 0.02 M, pH = 7.4). The globin chain (heme) concentration of the hemoglobin samples was 0.5 μM for the short (200−260 nm) wavelength scan and 5.0 μM for the long (260−470 nm) wavelength scan.

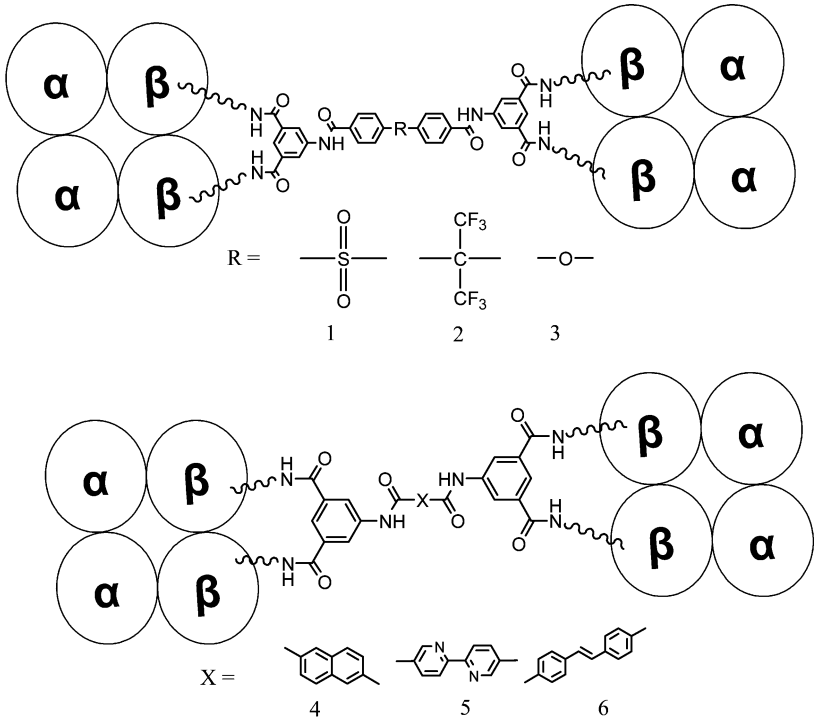
RESULTS

The syntheses of tetra-dibromosalicylate reagents (**1**−**3**) from three commercially available dicarboxylic acids ("**a-COOH**"), which were converted to acid chlorides. These were coupled with 5-aminoisophthalic acid to form amides via their acid chlorides ("**a-COCl**"). The resulting derivatives ("**b-COOH**") provide sites for producing cross-links by conversion of their four carboxyls to esters of 3,5-dibromosalicylic acid. These were converted to tetrakis-carbonyl chlorides and then to the cross-linking reagents, tetrakis-dibromosalicylates (**1**−**3**), using previously reported procedures (*20*) (Scheme 1). The other three tetrakis-dibromosalicylates (**4**−**6**) were synthesized from the tetrakis-carboxylic acids ("**b-COOH**") (Scheme 2) described previously (*23, 24*).

Two equivalents of a cross-linking reagent modified at least 95% of one equiv of native deoxyHb (pH 9.0 at 37 °C), except for reagents **4** and **6**, which left around 30% of the hemoglobin unmodified. No cross-linked products were observed in the reactions using hemoglobins in CO- or oxygen-bound forms. These large reagents target the BPG-binding site of Hb, which is not accessible to them when the protein is in the R state.

The separation of hemoglobin mixtures through a size-exclusion column under partially dissociating conditions gave two major components at 33 and 37 min retention times (Figure 1), both of which eluted ahead of native hemoglobin and un-cross-linked hemoglobins at 40 min. The yields of the largest molecular mass components (~128 kDa), which elutes at 33 min, varied from 26% to 42% (Table 1). Reagent **1**, with a central sulfone, gave the highest yield of a ~128 kDa material (Table 1).

Scheme 3: Proposed Structure of Cross-Linked Hemoglobin Bis-tetramers



Globin chain analysis (*32*) through C4 reverse-phase HPLC showed that only one component derived from native hemoglobin had been modified (Figure 2, solid line).

SDS−PAGE (*23*) analysis of native hemoglobin produces a band at ∼16 kDa (Figure 3, lane 2), while the purified 128 kDa material gave bands at ∼16 and ∼64 kDa.

Digest mapping of the modified globin chains compared to native globin chains indicates the sites of modified amino groups. The decrease of peaks from fragments corresponding to *β*T-1a′ (*β*1−7), *β*T-10a′ (*β*83−90), and *β*T-9 (*β*67−82) indicated that there were modifications to the amino group of the *β* chain N-terminal valine and/or the *ε*-amino group of *β*′-Lys-82. This modification pattern was consistent with the specificity of their parent isophthalyl dibromosalicylate *N*,*N*′-bis[bis(3,5-dibromosalicyl)isophthalyl]terephthalamide (DBIT) (*20*) and counterparts isophthalyl phosphastes (*23, 24*) that produced cross-linked hemoglobin bis-tetramers.

The hydrolysates after trypsin−Glu-C digest of native and modified *β* globin chains were mapped by both C18 reverse-phase HPLC and MALDI-TOF mass spectroscopy. HPLC analysis showed that only three peptide fragments were decreased in globin chains from the purified 128 kDa material (Figure 4). When the fragments were monitored at 258 nm, where isophthalamides (derivatives of the cross-linking reagents) absorbed, the hydrolysates from the digested native globin chains were not detected. On the other hand, we observed one peak from the hydrolysates of the modified proteins at that wavelength (Figure 4, dashed line).

Circular dichroism spectra obtained from 200 to 260 nm (Figures 5 and 7) were very similar for the native and 128 kDa molecular mass products in both deoxygenated and oxygenated states, while those in 260−470 nm region (Figures 6 and 8) showed differences.

The oxygen-binding curves and corresponding Hill plots of the six 128 kDa molecular mass products along with those of native hemoglobin are presented in Figures 9, 10, and 11. The P_{50} and n_{50} values are summarized in Table 2. In general, cross-linked bis-tetramers **1**−**3** have increased oxygen affinities compared to native hemoglobin and Hill coefficients for oxygenation that are similar to that of native hemoglobin. On the other hand, cross-linked bis-tetramers **4**−**6** interacted with oxygen with decreased affinities and Hill coefficients compared to native hemoglobin.

DISCUSSION

Previous work with acyl phosphate (*37, 38*) and dibromosalicylate (*20, 39, 40*) reagents revealed that dibromosalicylates are less prone to hydrolysis than the related acyl phosphate derivatives. Since partial hydrolysis will lead to a more complex mixture of products, we chose to use dibromosalicylates to minimize partial destruction of the reactive portion of the reagents. The synthesis of acyl dibromosalicylate derivatives, which was achieved by reacting activated carboxylic acids with protected dibromosalicylic acid, gave useful yields of reagents (*20*). Although the acyl dibromosalicylates are less reactive than acyl phosphates and have additional steric limitations in their reactivity patterns, they efficiently cross-linked hemoglobins as bis-tetramers (*20*). Therefore, we used tetrakis-dibromosalicylates (*20*) as the cross-linking reagents for this study. These new tetrakis-dibromosalicylate cross-linking reagents were readily synthesized from commercial dicarboxylic acids, which were efficiently extended to relevant tetrakis-carboxylic acid precursors.

Modifications of deoxyhemoglobin by the newly synthesized cross-linking reagents were carried out in solutions at pH 9.0 (*20*), where the carboxylate groups (pK_a ∼ 4) of the dibromosalicylates electrostatically direct reaction to the collection of protonated amine side chains of hemoglobin that bind 2,3-BPG (*41*). Transient proton movements between the carboxyl and amino groups facilitate acylation of the amino groups of the lysyl side chains (*41*).

Reactions using different cross-linking reagents gave cross-linked hemoglobins as mixtures with different molecular mass compositions. Reagent **1** produced the 128 kDa product with the greatest efficiency (42%). Their mass of 128 kDa corresponded to four $\alpha\beta$ dimers covalently linked as a cluster, which we designated as being "cross-linked hemoglobin bis-tetramers". The other major products (~64 kDa) in the reactions were cross-linked hemoglobin tetramers. Because our objective is to create stable assemblies that are larger than cross-linked tetramers, we focused on the properties of the three cross-linked hemoglobin bis-tetramers.

Scheme 3 shows the proposed structures for the cross-linked hemoglobin bis-tetramers **1**−**3** with angular intertetrameric structures and another three bis-tetramers **4**−**6** with rigid central structures for comparison.

Cross-linked hemoglobin bis-tetramers with various intertetrameric linkage structures (Scheme 3) behaved remarkably differently with respect to binding oxygen. The results are summarized in Table 3. We observed a significant decrease in cooperativity (n_{50} under 2.0) with bis-tetramers **4**−**6**. On the other hand, bis-tetramer **1**, with the sulfone central linkage, binds oxygen with a level of cooperativity equal to that of native hemoglobin ($n_{50} = 2.7$), while bis-tetramers **2** ($n_{50} = 2.2$) and **3** ($n_{50} = 2.5$) also show much higher cooperativity than **4**−**6**.

Introduction of a single sp^3 center into the structure linking the two tetramers produced significant increases in cooperativity in oxygen binding in **1**−**3** compared to their linear analogues. Therefore, intertetrameric links with a torsional flexibility and nonlinearity introduced by the tetrahedral center remarkably enhanced oxygen-binding cooperativities of bis-tetramers. It is likely that this is the result of changing the overall shape and flexibility of the assembly of tetramers. Moreover, the bis-tetramers **1**−**3** possess higher oxygen affinities than the native protein and **4**−**6**. Previous studies had shown that hemoglobins cross-linked in deoxygenated states tended to bind oxygen with decreased affinities (*34*). This had been considered to be an appropriate property for acellular oxygen carriers (*1*).

However, the potential advantage of high oxygen affinity in hemoglobin derivatives used as oxygen carriers has been presented by Winslow and co-workers using mathematical models of microcirculation through capillaries (*41*). They conclude that a high oxygen affinity combined with cooperativity will enhance the effectiveness of oxygen delivery to tissues, avoiding early release into nonhypoxic areas, leading to a homeostatic response that decreases blood flow. With a combination of high affinity and high cooperativity, oxygen transport capacity is increased in regions with lower oxygen tension. This allows products to be manufactured with lower concentrations of protein, decreasing the chances of side effects. This analysis led to a reevaluation of the desirable properties for a safe and effective HBOC. These include increased oxygen affinity and high binding cooperativity, as well as defined structures, increased molecular size, and increased viscosity (*41*). The three bis-tetramers developed in this work possess all of the proposed properties, which make them very promising as potential HBOCs.

Circular dichroism has been applied to study the protein folding and microenvironment around hemes within different hemoglobin species (*36*). CD spectra from 200 to 260 nm reveal the secondary structures of globin chains, while spectra obtained at longer wavelength, from 260 to 470 nm, reflect the microenvironment around hemes embedded on globin chains. The consistency of the spectra from 200 to 260 nm reveal that hemoglobin chains are properly folded after modification. On the other hand, spectra from 260 to 470 nm differentiate among species, which shows the altered microenvironment around hemes in cross-linked hemoglobin bis-tetramers. This observation explains the changes of oxygen-binding properties after modification. In addition, native hemoglobin and bis-tetramers all gave significantly different spectra from 260 to 470 nm between deoxygenated and oxygenated states. Because changes of the microenvironment around hemes while binding oxygen account for the positive oxygen-binding cooperativity of native hemoglobin, the same trends found with bis-tetramers are consistent with their high oxygen-binding cooperativities.

The introduction of a single sp^3 center in the intermolecular connection allowed the full cooperativity of hemoglobin to be restored as measured by the Hill coefficient. The high oxygen affinity was associated with other modified hemoglobins containing a cross-link between β chains with the isophthalyl linker at lysine 82 of each subunit. However, the planar interprotein linkers we previously examined led to a nearly complete loss of cooperativity. These results suggest that steric interactions between the tetramers in the planar-linked cases are interfering with the subunit motions that are responsible for cooperativity while the introduction of the more flexible connection, made possible by the tetrahedral center, permits the tetramers to become separated. Molecular mechanics modeling of the linkers shows that the lowest energy conformation in the system without an sp^3 center places the linked sites in a direct linear relationship along the linker. A similar linker that contains an sp^3 center necessarily attains a conformation with a sharp bend at that center, leading to an arrangement in which the linked sites are related by an out of plane angle of about 30°. This appears to remove steric restrictions and permits the motions associated with cooperativity (*42*).

CONCLUSION

Three bis[bis(3,5-dibromosalicyl)isophthalyl] derivatives were designed and synthesized with flexible central bridge structures. The resulting bis-tetramers contained single molecular size components that were modified on four β chains at β-Val-1 and/or β'-Lys-82. Unlike less flexibly connected bis-tetramers, these materials showed high levels of cooperativity in oxygen binding. Spectroscopic examination showed that the effects were related to the heme environment. We expect that the properties of these materials can be used to evaluate the importance of size and cooperativity in specific structures when evaluating potential approaches to safe and effective alternatives to red cells in transfusion. The results are striking in that a small modification within a cross-linker has important dynamic implications for the entire modified protein.

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