Hemoglobin bis-tetramers via cooperative azide-alkyne coupling.

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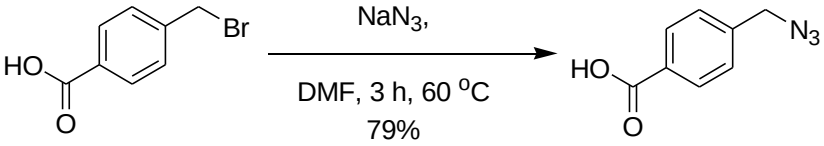
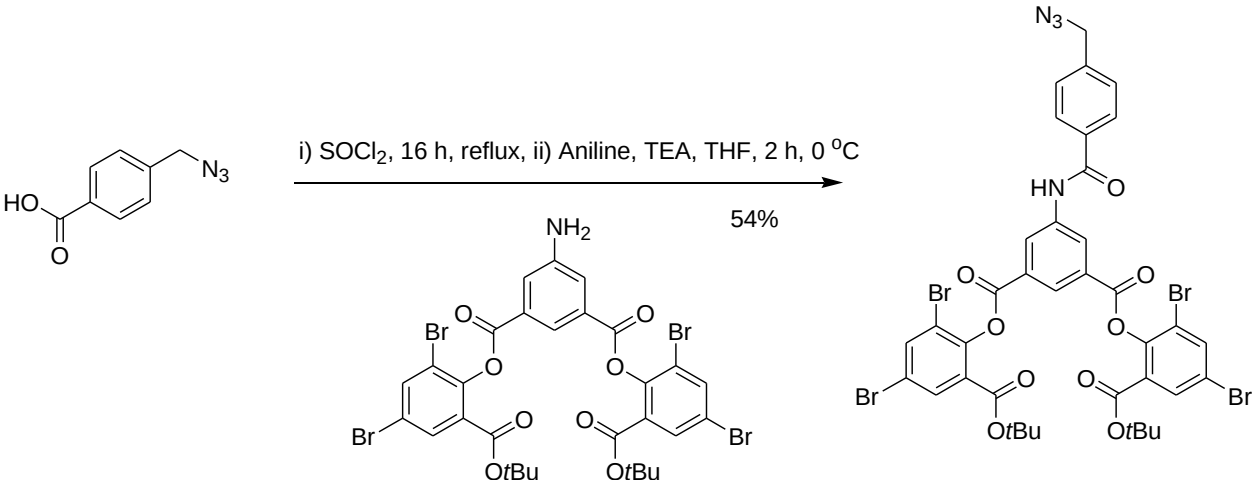
1. Experimental

4-Azidomethyl-benzoic acid



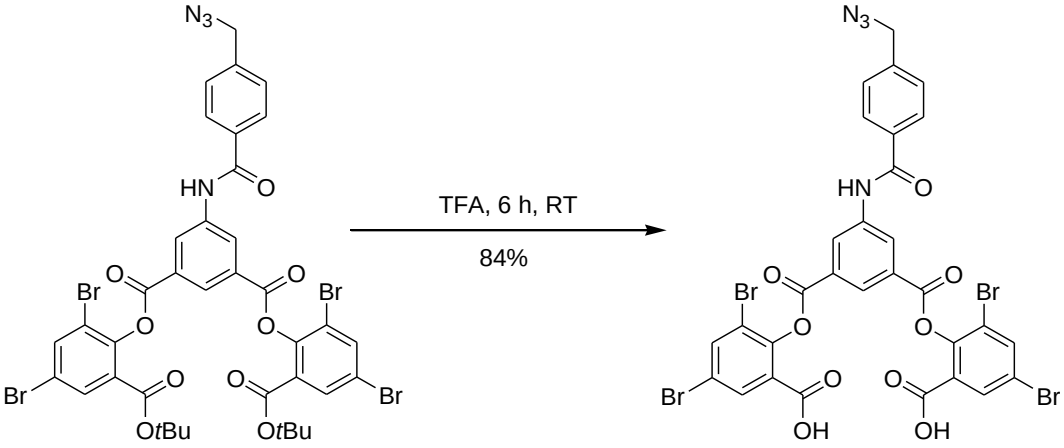
To a stirred solution of α-bromotoluic acid (0.645 g, 3 mmol) in DMF (3 mL) was added sodium azide (0.215 g, 3.3 mmol). The reaction mixture was then heated for 3 hours at 60 ^{o}C until tlc analysis indicated no remaining starting material. Reaction vessel was allowed to cool to room temperature and water (20 mL) was added. Resultant precipitate was filtered and dried at 60 ^{o}C for 1 hour to afford the title compound as a fine white solid (0.418 g, 79%). ^{1}H-NMR (300 MHz, DMSO) δ ppm: 4.53 (2H, s), 7.47 (2 H, d, J = 8.4 Hz), 7.94 (2 H, d, J = 8.4 Hz), 12.95 (1 H, br s); LR-ESI: 176 $[M-H]^-$, 177 $[M]^-$; HR-ESI: $[M-H]^-$ $C_8H_6N_3O_2$ requires 176.0450, found 176.0454 (error = -2.5 ppm).

5-(4-Azidomethyl-benzoylamino)-isophthalic acid bis-(2,4-dibromo-6-tert-butoxycarbonyl-phenyl) ester



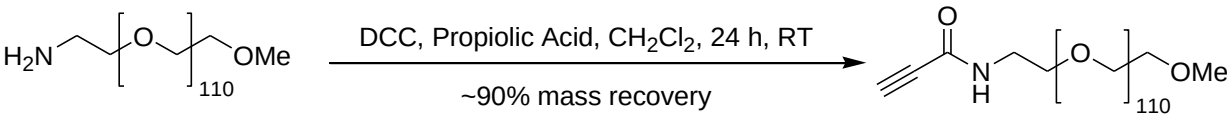
A stirred solution of 4-azidomethyl-benzoic acid (0.089 g, 0.5 mmol) in thionyl chloride (2 mL) was heated at reflux for 16 hours. Excess thionyl chloride was removed under vacuum and the crude acid chloride taken up in THF (2 mL). This solution was then introduced dropwise under nitrogen to a stirred solution of 5-amino-isophthalic acid bis-(2,4-dibromo-6-*tert*-butoxycarbonyl-phenyl) ester (0.423 g, 0.5 mmol) and triethylamine (83 μL, 0.6 mmol) in THF (2 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature over 2 hours, then quenched with water (20 mL). The organic component was then extracted with EtOAc (2 x 20 mL), washed with brine and dried over $MgSO_4$. Filtration and removal of the solvent *in vacuo* gave the crude product as a brown solid (0.49 g). Purification by flash chromatography, eluting in 15-20% EtOAc in n-hexane, afford the *title compound* as a white solid (0.27 g, 54%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 1.40 (18 H, s), 4.45 (2 H, s), 7.44 (2 H, d, J = 8.1 Hz), 7.90-7.96 (4 H, m), 8.01 (2 H, d, J = 2.4 Hz), 8.34 (1 H, br s), 8.83 (1 H, d, J = 1.5 Hz), 8.86 (2 H, d, J = 1.5 Hz); ^{13}C-NMR (300 MHz, $CDCl_3$) δ ppm: 28.19, 54.40, 83.46, 119.45, 119.84, 126.88, 127.93, 128.65, 129.04, 130.70, 133.88, 134.01, 139.06, 139.48, 146.76, 163.06, 162.60, 165.42 (2 signals not observed); LR-ESI: 1031 $[M+Na]^+$, 1026 $[M+NH_4]^+$

5-(4-Azidomethyl-benzoylamino)-isophthalic acid bis-(2,4-dibromo-6-carboxy-phenyl) ester, Reagent 1



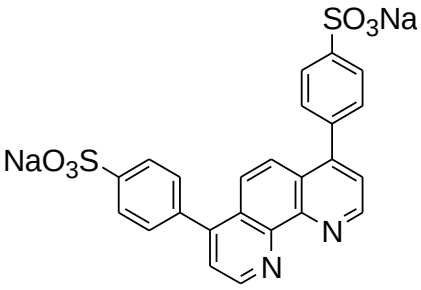
To a stirred solution of 5-(4-azidomethyl-benzoylamino)-isophthalic acid bis-(2,4-dibromo-6-*tert*-butoxycarbonyl-phenyl) ester (0.25 g, 0.25 mmol) in dichloromethane (8 mL) was added anhydrous trifluoroacetic acid (2 mL). The reaction mixture was stirred for 6 hours and the solvent removed *in vacuo* to give a yellow glass (0.27 g). The crude material was then taken up into the minimum amount of DMSO (~0.8 mL) and water (20 mL) added whilst stirring, generating a white precipitate. Solid thus formed was filtered and dried at 60 ^{o}C to afford the *title compound* as a fine white solid (0.188 g, 84%); ^{1}H-NMR (300 MHz, DMSO) δ ppm: 4.57 (2 H, s), 7.55 (2 H, d, J = 8.4 Hz), 8.07 (2 H, d, J = 8.4 Hz), 8.10 (2 H, d, J = 2.1 Hz), 8.33 (2 H, d, J = 2.1 Hz), 8.50 (1 H, t, J = 1.5 Hz), 9.03 (2 H, d, J = 1.5 Hz), 10.87 (1 H, s); IR cm^{-1}: 3074, 2359, 2101 (azide), 1738, 1717, 1699, 1197; LR-ESI: all [M-H]$^{-}$; 891 ($^{79}Br_4$ – HR run on this ion), 893 ($^{79}Br_3$,$^{81}Br_1$), 895 ($^{79}Br_2$,$^{81}Br_2$), 897 ($^{79}Br_1$,$^{81}Br_3$), 899 ($^{81}Br_4$); HR-ESI: [M-H]$^{-}$ $C_{30}H_{15}Br_4N_4O_9$ requires 890.7547, found 890.7577 (error = -3.4 ppm).

PEG alkyne, Reagent 2



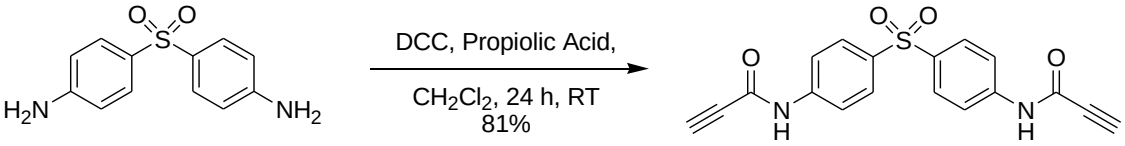
To a stirred solution of *O*-(2-aminoethyl)-*O*'-methylpolyethylene glycol 5000 (0.59 g, ~0.1 mmol) and propiolic acid (62 μL, 1 mmol) in dichloromethane (5 mL) at room temperature was added dicyclohexylcarbodiimide (0.206 g, 1 mmol) in one portion and the mixture allowed to stir for 24 hours. DCU by-product was remove by filtration, washing with dichloromethane, and the solvent removed in vacuo to give a sticky orange gum (~0.8 g). Gum was dissolved in DMF (~3 mL) with heating and stirred for 5 mins at room temperature to ensure all solid remained in solution. Diethyl ether (60 mL) was then added, generating a white precipitate. Solid thus formed was filtered and dried under vacuum to afford the title compound as a fine white powder (0.530 g) that was no longer ninhydrin active when analyzed by tlc. ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 2.86 (1 H, s), 3.36 (3 H, s), 3.39 (2 H, t, J = 5.7 Hz), 3.42-3.73 (m, PEG chain), 3.86 (2 H, t, J = 5.7 Hz), 6.79 (1 H, br s); IR cm^{-1}: 2887, 1963, 1660 (amide), 1343, 1110.

Bathophenanthrolinedisulfonic acid disodium salt, Reagent 3



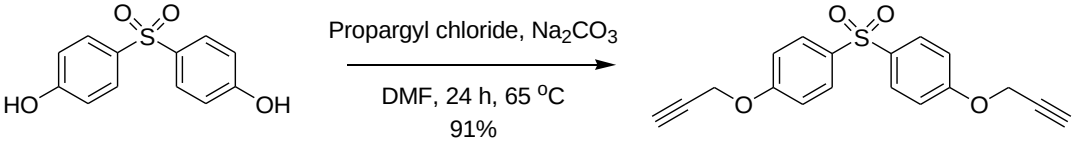
Reagent **3** was purchased from Alfa Aesar, cat. no. *B23244*, 98% purity

Propynoic acid [4-(4-propynoylamino-benzenesulfonyl)-phenyl]-amide, Reagent 4



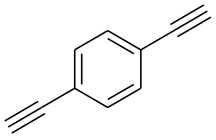
To a stirred solution of 4-aminophenyl sulfone (0.50 g, 2 mmol) and propiolic acid (0.55 mL, 8.8 mmol) in dichloromethane (50 mL) was added dicyclohexylcarbodiimide (1.65 g, 8 mmol) in one portion and the mixture allowed to stir for 72 hours. Resultant precipitate was filtered, washing with dichloromethane (20 mL) and dried under vacuum. Solid was then taken up into hot acetonitrile (40 mL) to dissolve product and the DCU by-product removed by filtration. Filtrate was then reheated and refiltered to remove residual DCU followed by removal of the solvent *in vacuo*, giving the crude product as a white solid (0.84 g). Purification by flash chromatography, eluting in 5-10% MeOH in dichloromethane, afforded the *title compound* as an off- white solid (0.57 g, 81%). ^{1}H-NMR (300 MHz, DMSO) δ ppm: 4.51 (2 H, s), 7.76 (4 H, d, J = 9.0 Hz), 7.87 (4 H, d, J = 9.0 Hz), 11.20 (2 H, s); LR-ESI: 353 $[M+H]^+$, 375 $[M+Na]^+$, 391 $[M+K]^+$; HR-ESI: $[M+H]^+$ $C_{18}H_{13}N_2O_4S$ requires 353.0604, found 353.0590 (error = 3.8 ppm).

Propargyl-4,4'-sulfonyldiphenol ether, Reagent 5



To a stirred solution of 4,4'-sulfonyldiphenol (0.25 g, 1 mmol) and sodium carbonate (0.31 g, 2.5 mmol) in DMF (2 mL) was added propargyl chloride (0.16 mL, 2.2 mmol). The reaction mixture was then heated at 65 ^{o}C for 24 hours until tlc analysis indicated no remaining starting material. Reaction vessel was allowed to cool to room temperature and water (20 mL) was added. Resultant precipitate was filtered and dried under suction to afford the title compound as a fine white powder (0.298 g, 91%); ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 2.54 (2 H, t, J = 2.4 Hz), 4.72 (4 H, d, J = 2.4 Hz), 7.03 (4 H, d, J = 6.9 Hz), 7.87 (4 H, d, J = 6.9 Hz); LR-ESI: 327 $[M+H]^+$; HR-ESI: $[M+H]^+$ $C_{18}H_{15}O_4S$ requires 327.0670, found 327.0685 (error = -4.7 ppm).

1,4-Diethynylbenzene, Reagent 6



Reagent **6** was purchased from Sigma Aldrich, cat. no. *632090*, 96% purity

2. Methods

Hemoglobin

The protein was purified from human red cells by the method described by Winslow *et al.*[1] Purified hemoglobin was stored in doubly distilled water at 0–5 ^{o}C. Concentrations of hemoglobin solutions were determined using the cyanomethemoglobin assay described by Salvati and Tentori.[2] The purity of hemoglobin was determined using the reverse-phase HPLC (RP HPLC) analysis described by Jones.[3]

SDS-PAGE analysis

The molecular weights of constituent proteins were estimated using polyacrylamide gel (10 or 12%, Tris-HCl) electrophoresis. Two dimensional Tris-HCl polyacrylamide gels were comprised of 10 or 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.[4] Globin chains were further denatured by heating at 100 ^{o}C for 15 min before loading onto the gel. Finished gels were stained with Coomassie Brilliant Blue. Further details are described in earlier papers.[5]

Analytical reverse-phase HPLC

A 330 A˚ pore-size C-4 Vydac column (4.6 × 250 mm) was used to separate modified globin chains. Gradient elution was initiated with 20% acetonitrile and run to 60% acetonitrile with 0.1% (v/v) TFA as developer.[3] The effluent was monitored at 220 nm.

Size exclusion HPLC

A Superdex G-200 (10 × 300 mm) column was used to investigate the size distribution of the modified protein mixtures. Samples were eluted under conditions that dissociate the hemoglobin tetramers into dimers (37.5 mMTris-HCl, pH 7.4, 0.5 M magnesium chloride).[6] The effluent was monitored at 280 nm.

3. Synthesis of cross-linked hemoglobin azide (HbN_3)

Reaction with deoxyhemoglobin (Scheme 2 in main article)

Carbonmonoxyhemoglobin (HbCO, 6 mL, ~12 μmol) was equilibrated through a Sephadex G-25 column (250 × 35 mm) into sodium borate buffer (0.05 M, pH 9.0) at 4 ^{o}C. The resultant solution (~30 mL) was oxygenated and deoxygenated to give deoxyhemoglobin (deoxyHb).[7] Reagent **1** (21 mg, 23.4 μmol) in DMSO (300 μL) was then introduced into the deoxyHb solution. The reaction was carried out for 20 h at 37 ^{o}C under nitrogen. Carbon monoxide was then passed over the modified hemoglobin for 15 min to protect the hemes. The modified HbCO was transferred to MOPS buffer (0.1 M, pH 7.2) through a Sephadex G-25 column (250 × 35 mm) at 4 ^{o}C. The mixture was concentrated (~14 mL), the concentration of the modified hemoglobin determined by UV-vis, and stored at 4

^{o}C. From this sample, a [0.1 mM] stock of HbN_3 in 0.02 M phosphate buffer (pH 7.4) was prepared, upon which all following studies were carried out.

Analysis of the synthesized material by HPLC using a G200 size exclusion column under partial disassociating conditions showed that a smooth conversion of hemoglobin had occurred to afford >90% of the desired cross-linked hemoglobin (see Figure SI 1(a)). Full analysis of the modified subunits using a C4 column under fully disassociating conditions confirmed that almost all of the β-subunits and a proportion of the α-subunits had been modified (Figure SI 1 (b)). Full interpretation of this chromatogram is included in the main article, as are the ESI mass assignments of the isolated BPG-ββ and αα peaks at 59 mins and 78 mins respectively. Comparison of the retention times of these isolated peaks with those of authentic BPG-ββ and αα cross-linked material (shown in Figure SI 2) helped further assign these components of the cross-linking reaction.

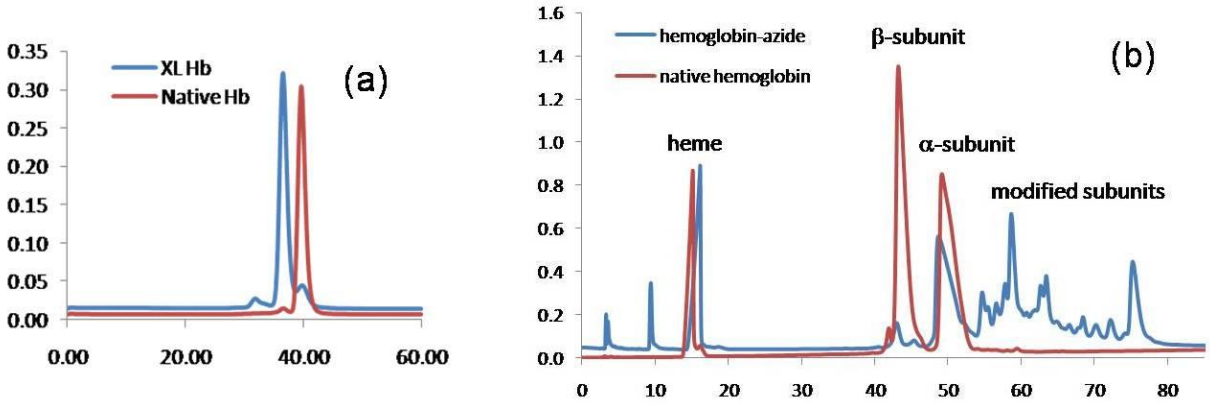


Figure SI 1. (a) G200 size exclusion HPLC. Peak at ~37 mins correlates with cross-linked, non-disassociated cross-linked HbN_3 tetramer (64 kDa); peak at ~40 min correlates with diassociated αβ protomer of native hemoglobin (32 kDa). (b) C4 HPLC showing almost complete modification of the β-subunits (blue line at 44 mins).

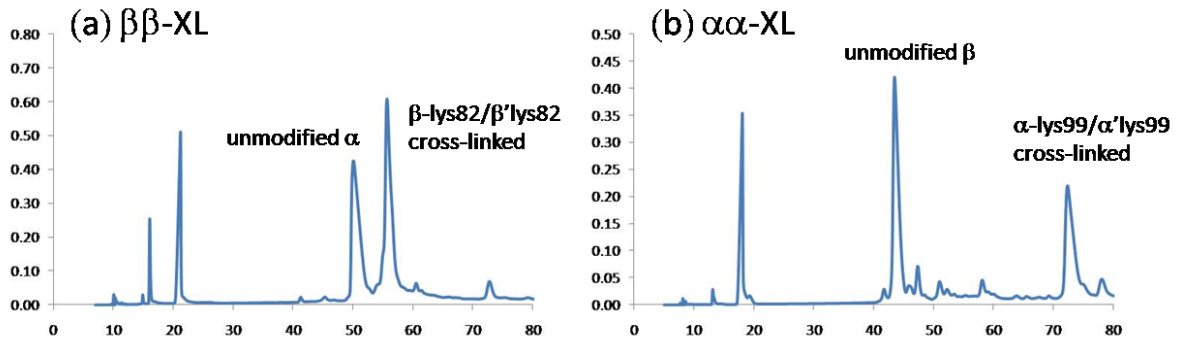


Figure SI 2. (a) C4 HPLC analysis of authentic BPG-ββ-TTDS cross-linked hemoglobin. (b) C4 HPLC analysis of authentic αα-fumaryl cross-linked hemoglobin.

4. Coupling of two HbN_3 proteins

To a 10 mL Agilent vial containing HbN_3 (2 mL of a 0.1 mM solution) was added Reagent 4 (20 μL of a 0.1 M solution in DMSO), Reagent 3 (60 μL of a 20 mM solution), $CuSO_4$ (100 μL of a 4 mM solution) and copper powder (2 mg). The vial was sealed and flushed with carbon monoxide, then shaken at room temperature for 8 hours. The modified HbCO was filtered and transferred to MOPS buffer (0.1 M, pH 7.2) through a Sephadex G-25 column (250 × 35 mm) at 4 ^{o}C. The mixture was concentrated and stored at 4 ^{o}C. Analysis by size exclusion HPLC (Figure 4 in main article) was carried out to quantify reaction and by SDS-PAGE (Figure SI 3) to confirm approximate size of the newly formed bis-tetramer.

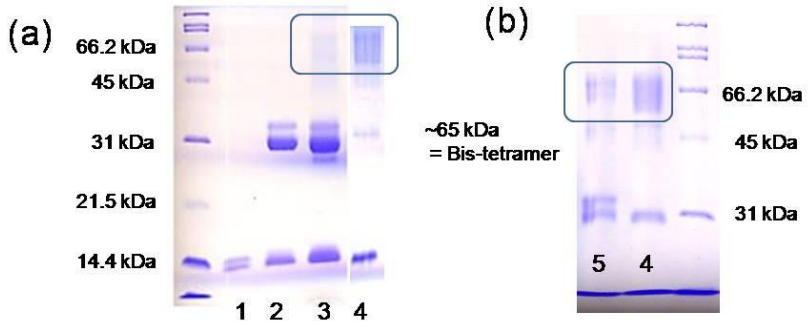


Figure SI 3. SDS-PAGE [(a) 12% & (b) 10%] analysis under reducing conditions; Lane 1: Native hemoglobin showing the dissociated α and β units around the 14.4 kDa marker, Lane 2: Cross-linked HbN_3 showing the unmodified α subunit at the 14 kDa marker and the αα and ββ-cross-linked species at the 31 kDa marker, Lane 3: Reaction of HbN_3 with reagent **4** after 8 hours; ββ-ββ bis-tetramer derived fragment appears as a diffuse band around the 66.2 kDa marker, Lane 4: Authentic amide-linked bis-tetramer synthesized by reported methods,[8] Lane 5: Purified (to about 85% purity) bis-tetramer run on a 10% gel.

5. PEG optimization controls

Reaction was carried out in a similar fashion to that outlined in the HbN_3 coupling reaction, using quantities detailed in the main article. Analysis of the control experiments is shown below:

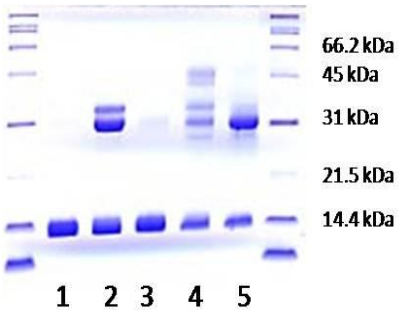


Figure SI 4. SDS-PAGE analysis under reducing conditions; Lane 1: Native hemoglobin showing the dissociated α and β units around the 14.4 kDa marker, Lane 2: Cross-linked HbN_3 showing the unmodified α subunit at the 14 kDa marker and the αα and ββ-crosslinked species at the 31 kDa marker, Lane 3: Native hemoglobin exposed to optimized conditions for 18 h, Lane 4: Cross-linked HbN_3 exposed to optimized conditions for 4 h, – band at 45 kDa is $HbPEG_1$,* Lane 5: TTDS-ββ-Hb exposed to optimized conditions for 18 h.

* Band appears ~10 kDa larger, rather than 5 kDa larger – this apparent higher mass is a typical observation when analyzing PEGylated proteins by SDS-PAGE.[9,10]

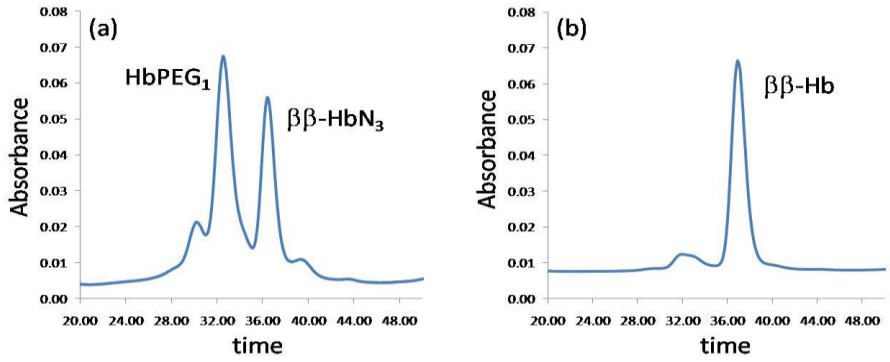


Figure SI 5. G200 analysis of control experiments; (a) Lane 4 – $HbPEG_1$ appears as a broad peak at 33 mins. (b) Lane 5 –un-modified bis-trimesyl-ββ-Hb appears at 37 mins.

6. Purification and analysis of unreacted material

Material from the reaction of HbN_3 with Reagent 4 was passed down a G100 size exclusion column under partially disassociating conditions to separate out bis-tetramer from unreacted/singly reacted HbN_3. Fractions containing bis-tetramer were combined to give a material that was ~85% pure bis-tetramer. This material was used to run the 10% SDS-PAGE gel in Figure SI 3. The remaining material from the column was combined and analyzed by C4 HPLC (Figure 5). Reintroduction of this material to the reaction conditions outlined in section 4 failed to produce any further bis-tetramer.

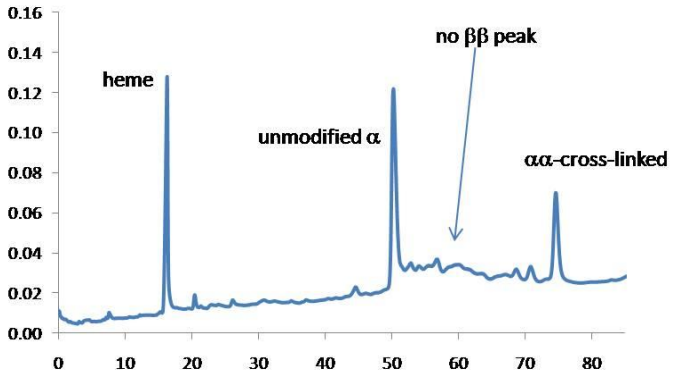


Figure SI 6. C4 analysis of unreacted/singly reacted components of the Hb-Hb coupling reaction

7. References

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