=Electronic Supplementary Information=

Core–shell clusters of human haemoglobin A and human serum albumin: artificial O_2-carriers having various O_2-affinities

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Experimental section

Purification of HbA from red blood cell (RBC)

Concentrated human red blood cell (RBC) suspension (15 mL) donated from the Japanese Red Cross Society was diluted with saline solution (15 mL) and centrifuged (4000 × *g*, 4 °C) for 10 min using a bench-top centrifuge (Allegra X-15R; Beckman Coulter Inc.). After removing supernatant, the equivalent saline solution was added to make the volume 30 mL. These dilution and centrifugation cycles were repeated three times. Then the obtained washed RBC ([Hb] = 4.7 mM, 19 mL) was poured slowly into deionized water (281 mL) and incubated for 1 h at 4 °C for haemolysis. The resulting dispersion was centrifuged (5000 × *g*, 4 °C) for 30 min to precipitate the insoluble stroma. The supernatant fluid (*ca.* 270 mL) was passed through stainless sieves (90 mm diameter, 100 μm and 45 μm pore size). Subsequently, the flow-through (aqueous HbA) was filtrated using a cellulose acetate membrane (76 mm diameter, 3.0 μm pore size) (Advantec Toyo Kaisha Ltd.) in a pressure holder (KST-90) with subsequent filtrations using 0.8 μm, 0.45 μm, and 0.2 μm pore-size membranes in order. Later, this HbA solution was concentrated to 90 mL using a tangential-flow filtration system with a Minimate TFF Capsule (Omega 10K membrane; Pall Corp) and was transferred into a 100-mL glass flask sealed with a rubber septum. After the CO gas was purged into the flask to generate carbonyl HbA, the solution was heated at 65 °C for 10 h with gentle stirring in the dark. Then the resultant mixture was centrifuged (5000 × *g*, 4 °C) for 20 min to remove denatured proteins. Finally, the red supernatant was dialyzed using a membrane tube (6–8 kDa MWCO, Spectra/Por 1; Spectrum Laboratories Inc.) against 150 volumes of deionized water at 4 °C. The purified HbA sample was analysed using a Native-PAGE and size exclusion chromatography (SEC) to confirm the protein integrity (purity >99.9%). The SEC system consisted of an HPLC (LaChrom Elite; Hitachi High-Technologies Corp.) with a column (Shodex Protein KW-803; Showa Denko K.K.). PB (50 mM, pH 7.4) was used as the mobile

phase at 25 °C. The Hb concentration was assayed ([HbA] = 4.4 g/dL) using a Nescoat Hemokit-N (Alfresa Pharma Corp.). The yield of Hb was 75% based on the washed RBC. The purified carbonyl Hb was preserved at 4 °C under CO atmosphere.

Purity measurement of HbA

To assay the content of residual stromal lipid components in the purified HbA, lipid extraction was conducted according to our previously reported protocol.[19] The removal efficiency of phospholipid was calculated as greater than 99.99% from the ratio of the corresponding peak areas in purified HbA and RBC.

Maleimide group assay on HbA

To the PBS solution (pH 7.4) of SMCC-HbA (4.45 μM, 10 mL) in the glass bottle (20 mL volume), 2-mercaptoetahol (2ME) (6.74 mM in PBS, 148 μL) was added, and the mixture was stirred at 200 rpm. After 30 min, 2 mL of the solution was transferred into the quartz cuvette, and 4,4'-ditiopyridine (25 mM in PBS, 80 μL) was added, subsequently incubating for 30 min under the dark at 25 °C. The absorbance of the solution at 324 nm, which is based on 4-thiopyridinone, yielded the remaining thiol concentration. The decreased 2ME concentration corresponded to the maleimide concentration in the sample. If the sulfhydryl group of Cys-93(β) remains, it should be subtracted.

Results

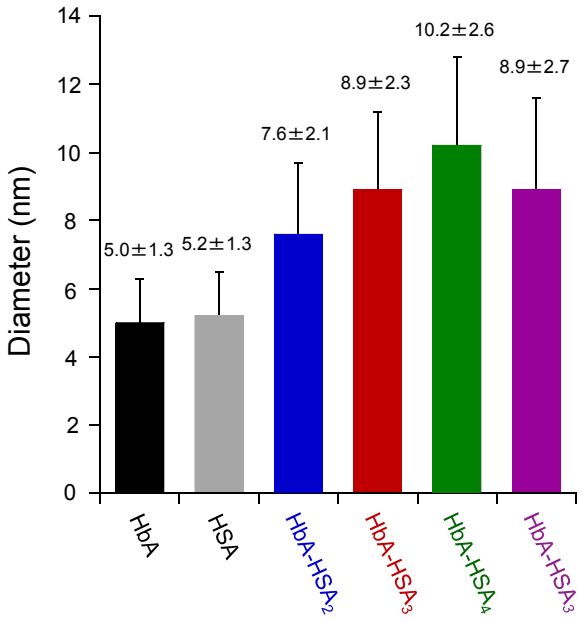

Fig. S1 Diameters of HbA-HSA$_m$ (m = 2, 3, 4), HbA-HSA$_3$, HbA, and HSA measured by DLS.

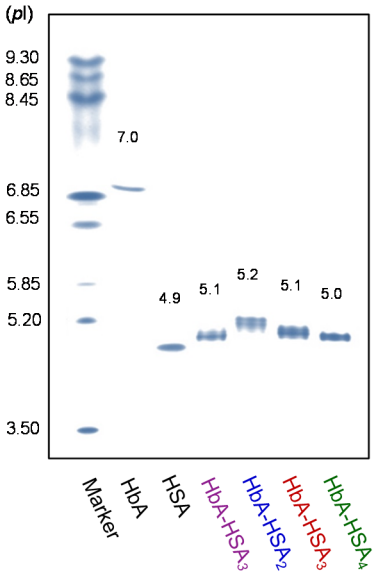

Fig. S2 Isoelectric focusing patterns of HbA-HSA$_m$ (m = 2, 3, 4), HbA-HSA$_3$, HbA, and HSA.

Movie S1 [Open the attached wmv file (MovieS1)] Spatial molecular view of HbA-HSA_3 cluster. Colour code: Hb, red; HSAs, chartreuse, lemon, and pale green. Hemes (C, grey) in HbA, Cys-34 (S, yellow) in HSA, and crosslinking moieties (C, green) are shown in space-filling representations. PDB ID: 2DN3 for HbA, 1E78 for HSA.

Table S1 Diameter measured by DLS and *p*I value of HbA-HSA_m clusters

Haemoproteins	Diameter (nm)	*p*I
HbA-HSA_3	8.9 ± 2.7	5.1
HbA(T)-HSA_3	8.8 ± 2.3	5.1
$\alpha\alpha$HbA(T)-HSA_3	8.9 ± 2.9	5.1

Table S2 UV-vis absorption spectral data of HbA-HSA_m clusters in PBS solution (pH 7.4) at 25 °C

	λ_{max}(nm)		
Haemoproteins	deoxy	oxy	carbonyl
HbA-HSA_2	429, 556	414, 541, 576	419, 538, 569
HbA-HSA_3	430, 556	414, 541, 577	420, 538, 569
HbA-HSA_4	430, 556	413, 541, 577	419, 538, 569
HbA-HSA_3	429, 556	413, 540, 576	419, 538, 568
HbA(T)-HSA_3	430, 555	414, 542, 577	420, 538, 569
$\alpha\alpha$HbA(T)-HSA_3	430, 554	414, 542, 577	419, 538, 569
HbA	430, 555	414, 541, 577	420, 539, 569
HbA[a)]	430, 555	415, 541, 577	419, 540, 569

[a)] From ref 37.