Supporting Information

Supramolecular Hemoprotein–Gold Nanoparticle Conjugates

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Experimental Procedures

Instruments.

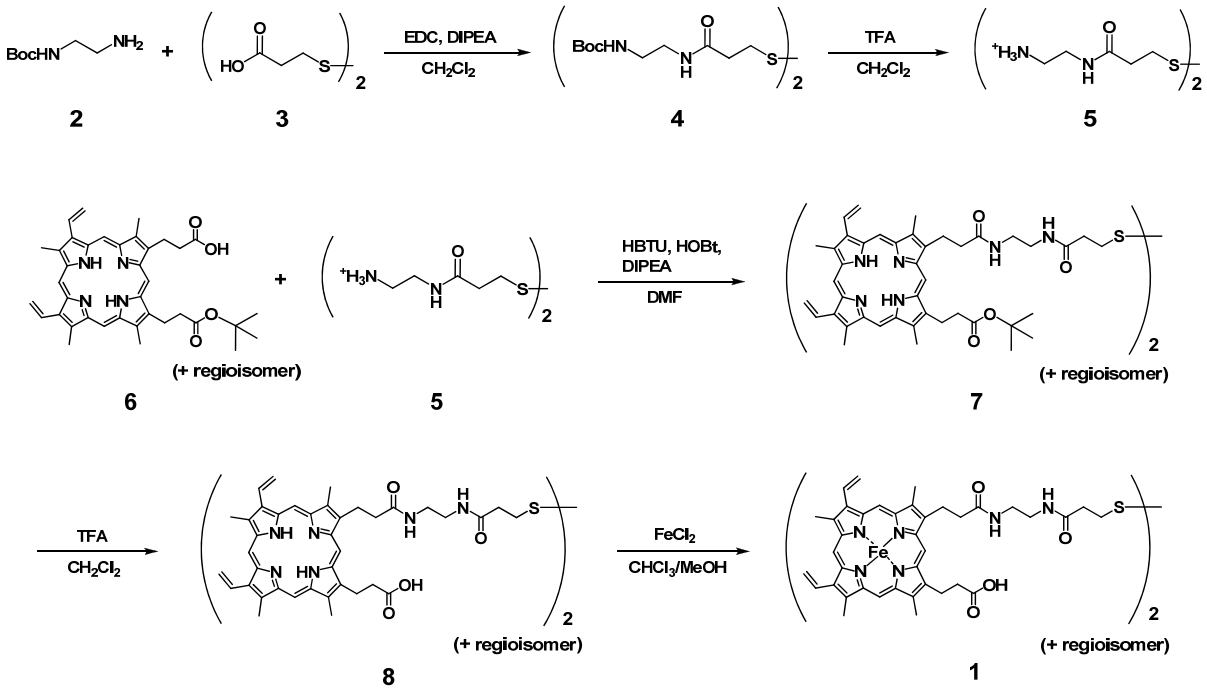
^{1}H NMR spectra (400 MHz) were recorded on a Bruker DPX400 NMR spectrometer. ^{1}H NMR chemical shift values are reported in ppm relative to the residual solvent resonances. ESI MS analyses were performed with a TOF mass spectrometer on an Applied Biosystems Mariner API-TOF Workstation. UV-vis spectra were taken by a SHIMADZU UV-3150 or UV-2550 spectrophotometer equipped with a thermostated cell holder. The pH values were monitored by an F-52 Horiba pH meter. Size exclusion chromatographic (SEC) analyses were performed using a Superdex 7510/300 GL column with an ÄKTA FPLC system and a fraction collector Frac-920 (GE Healthcare). The analyses were performed at 4 ºC using a

flow rate of 0.5 mL min^{-1} with the detection at 405 nm. Transmission electron microscopy (TEM) measurements were performed using H-7650 Zero A (Hitachi High-Technologies). Atomic force microscopy (AFM) measurements were taken by a MFP-3D-SA (Asylum Research). The size of nanoparticles was determined by dynamic light scattering using nano Partica SZ-100 (Horiba Scientific). The scanning electron microscopy (SEM) was performed using JEOL JSM-6701F.

Materials.

Protoporphyrin IX mono *t*-butyl ester (**6**),[S1] mono-*N*-Boc protected ethylenediamine (**2**),[S2] and 4,4'-dithiodipropanoic acid (**3**)[S3] were prepared according to the procedures reported in the literatures. Other reagents and chemicals were purchased and used as received. The H63C mutant of cytochrome b_{562} (cyt b_{562}) was expressed in *E. coli* and purified as previously reported.[S4]

Scheme S1. Synthesis of heme dimer with disulfide linkage



3,3'-Dithiobis[*N*-(2-*tert*-butoxycarbonylaminoethyl)propanamide] (4). To a mixed solution of **2** (152 mg, 9.5 x 10^{-4} mol) and **3** (100 mg, 4.8 x 10^{-4} mol) in 20 mL of CH_2Cl_2 was added *N*,*N*-diisopropylethylamine (DIPEA) (170 μL, 9.8 x 10^{-4} mol) in an ice bath under N_2 atmosphere. To the solution, kept cooling for 30 min, was added EDC (182 mg, 9.5 x 10^{-4} mol) in 20 mL of CH_2Cl_2 at 0 ºC and the reaction mixture was stirred for further 18 h at room temperature. After the reaction mixture was washed with 10% citrate (5 × 50 mL), $NaHCO_3$ aq. (5 × 50 mL), and water (5 × 50 mL), the organic phase was dried over Na_2SO_4 and evaporated to give **4** (140 mg, 60%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.76 (d, 2H, *J* = 5.2 Hz)), 5.18 (d, 2H, *J* = 5.6 Hz), 3.38 (td, 4H, *J* = 5.5 Hz, *J* = 5.2 Hz), 3.28 (td, 4H, *J* = 5.5 Hz, *J* = 5.6 Hz), 2.99 (t, 4H, *J* = 7.2 Hz), 2.58 (t, 4H, *J* = 7.2 Hz), 1.44 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 157.0, 79.7, 40.6, 40.2, 35.9, 34.6, 28.4; IR (KBr)/cm^{-1} 3339, 3303, 2980, 1690, 16545, 1645; HRFABMS (positive mode) *m/z* calcd for $C_{20}H_{38}N_4O_6S_2$ $(M + H)^+$ 495.2313, found 495.2329.

3,3'-Dithiobis[*N*-(2-aminoethyl)propanamide] (5). To a solution of **4** (100 mg, 2.0 x 10^{-4} mol) in 35 mL of CH_2Cl_2 was added 6 mL of TFA under N_2 atmosphere and the reaction mixture was stirred for 30 min at room temperature. The solvent was then evaporated to give **5** (79 mg, 96%) as a colorless oil: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.20 (t, 2H, J = 5.6 Hz), 7.87 (t, 6H, J = 6.0 Hz), 3.28 (td, 4H, J = 6.4 Hz, J = 5.6 Hz), 2.89 (t, 4H, J = 7.2 Hz), 2.85 (qt, 4H, J = 6.0 Hz, J = 6.4 Hz), 2.50 (t, 4H, J = 7.2 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.5, 48.1, 46.0, 44.5, 42.9; IR (neat)/cm^{-1} 3276, 1778, 1673; HRFABMS (positive mode) *m/z* calcd for $C_{10}H_{22}N_4O_2S_2$ $(M + H)^+$ 295.1264, found 295.1279.

Protoporphyrin IX dimer 7. To a solution of **6** (200 mg, 3.2 x 10^{-4} mol), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (123 mg, 3.2 x 10^{-4} mol) and 1-hydroxybenzotriazole (43 mg, 3.2 x 10^{-4} mol) in 15 mL of DMF was added DIPEA (120 μL, 6.9 x 10^{-4} mol) and then **5** (42 mg, 8.1 x 10^{-5} mol) in 10 mL of DMF in dropwise fashion. The reaction mixture was stirred for 18 h at room temperature. The mixture was diluted with CH_2Cl_2 and the organic layer was washed with 5% citrate (3 x 50 mL), $NaHCO_3$ aq. (3 x 50 mL), water (3 x 50 mL) and dried over Na_2SO_4. The solution was concentrated and subjected to a silica gel column chromatography ($CHCl_3$/MeOH = 40/1, v/v). The collected fraction was evaporated to give **7** (40 mg, 33%) as a purple solid: ^{1}H NMR (400 MHz, pyridine-d_5) δ 10.2–9.9 (m, 9H), 8.27–8.24 (m, 4H), 6.34–6.30 (m, 4H), 6.12–6.10 (m, 4H), 4.40–4.31 (m, 8H), 3.52–3.26 (m, 40H), 2.78–2.70 (m, 4H), 2.20–2.10 (m, 4H), 1.24 (s, 18H); HRFABMS (positive mode) *m/z* calcd for $C_{86}H_{102}N_{12}O_8S_2$ $(M + H)^+$ 1495.7468, found 1495.7482; UV-vis (MeOH)) λ_{max} / nm (absorbance) 664 (0.013), 633 (0.012), 577 (0.018), 540 (0.024), 505 (0.031), 393 (0.33).

Protoporphyrin IX dimer 8. To a solution of **7** (40 mg, 2.7 x 10^{-5} mol) in 20 mL of CH_2Cl_2 was added 4 mL of TFA and the reaction mixture was stirred for 7 h at room

temperature. The solvent was evaporated and the residue was washed with ether to give **8** (29 mg, 80%) as a purple solid: ^{1}H NMR (400 MHz, pyridine-d_5) δ10.0–9.82 (m, 9H), 8.33–8.23 (m, 4H), 6.36–6.28 (m, 4H), 6.11–6.08 (m, 4H), 4.37–4.33 (m, 8H), 4.30–4.25 (m, 4H), 3.60–3.28 (m, 40H), 2.81–2.80 (m, 4H), 2.20–2.15 (m, 4H); HRFABMS (positive mode) *m/z* calcd for $C_{78}H_{86}N_{12}O_8S_2$ $(M + H)^+$ 1383.6216, found 1383.6191; UV-vis (MeOH) λ_{max} / nm (absorbance) 628 (0.0077), 577 (0.015), 540 (0.020), 508 (0.024), 404 (0.30).

Heme dimer 1. Protoporphyrin IX **8** (29 mg, 2.1 x 10^{-4} mol), ferrous chloride hydrate (150 mg, 7.5 x 10^{-4} mol), and $NaHCO_3$ (10 mg) were dissolved in an N_2-purged $CHCl_3$/MeOH solvent at ratio of 2/1 (40 mL) and the mixture was refluxed for 3 h. After exposure to the air, the solution was evaporated to dryness. The residue was washed with 0.1 M HCl followed by ether to give **1** (25 mg, 80%) as a dark brown solid: HRFABMS (positive mode) *m/z* calcd for $C_{78}H_{82}Fe_2N_{12}O_8S_2$ $(M + H)^+$ 1490.4519, found 1490.4480; UV-vis (pyridine) λ_{max} / nm (absorbance) 555 (0.013), 522 (0.014), 407 (0.12).

Preparation of proteins.

Apo-cyt b_{562}. Removal of the native heme from wild-type cytochrome b_{562} (wt-cyt b_{562}) to obtain apo-cyt b_{562} was carried out according to Teale's 2-butanone method.[S5] The pH value of the solution of cyt b_{562} and 100 mM histidine was adjusted to pH 1.8 with the dropwise addition of 0.1 M HCl. The hemin was extracted by 2-butanone four times. The solution of apo-cyt b_{562} was then dialyzed against 1 L of 50 mM Tris-HCl buffer (pH 7.3).

Cyt b_{562} polymer (poly-cyt b_{562}). A large amount of the H63C protein was oxidized during purification to give the H63C dimer with disulfide linkage between the proteins. To cleave the disulfide bond in the H63C protein, a solution of H63C (2.4 x 10^{-3} mol) in 200 μL of 50 mM Tris-HCl buffer (pH 7.3) was added to 10 mM DTT solution in 3 mL of 50 mM Tris-HCl buffer (pH 7.3). The excess DTT was then removed by ultrafiltration and gel

filtration using a Hi-Trap desalting column (5 ml, G.E. Healthcare). Next, to the solution of the reduced H63C (3.4 mL) was added the synthetic maleimide heme[S6] (1.6×10^{-6} mol, 6.7 equiv.) in 600 μL of DMSO. The reaction mixture was stirred for 2 h at room temperature. The solution was acidified to pH 1.8 by HCl solution and the free heme groups (the native heme and the unreacted modified one) were removed from the solution by 2-butanone extraction five times. The separated aqueous phase was dialyzed against a 100-fold volume of 50 mM Tris-HCl buffer (pH 7.3) to obtain apo-cyt b_{562} unit having the heme moiety on the surface. The sample solution was concentrated by ultrafiltration to 2.8 mM and was stored in dark at –20 ºC. The UV-vis spectra of the apo-cyt b_{562} solution showed the characteristic Soret (418 nm) and Q (530 and 564 nm) bands which are consistent with those of the wild-type ferric cyt b_{562}. This results support the fact that the external heme on the surface was incorporated into the heme pocket of the protein matrix to form H63C polymer (poly-cyt b_{562}). Successful preparation of the polymeric form of cyt b_{562} was confirmed by size exclusion chromatography and native PAGE analysis.

Apo-dimer-cyt b_{562}. The dimeric H63C cyt b_{562} (dimer-cyt b_{562}) via an interprotein disulfide bond was purified by size exclusion chromatography (SEC) using a Superdex 7510/300 GL column with a elution of 50 mM Tris-HCl buffer (pH 7.3) containing 100 mM NaCl (flow rate: 0.5 mL/min, 4 ºC, detection: 405 nm). Removal of the native heme from the purified H63C dimer was carried out according to Teale's 2-butanone method.[S5] The solution containing H63C dimer and 100 mM histidine was adjusted to pH 1.8 by the dropwise addition of 0.1 M HCl, followed by extraction of the hemin was done by 2-butanone four times. The solution of apo-dimer-cyt b_{562} was dialyzed against 1 L of 50 mM Tris-HCl buffer (pH 7.3) to give purified protein. MALDI-TOF MS (positive mode) *m*/*z* calcd for $C_{1010}H_{1652}N_{290}O_{336}S_8$ $[M + H]^+$ 23491, found 23497.

Preparation of gold nanoparticles

Gold nanoparticles with an approximate average diameter of 15 nm were prepared by reduction of $HAuCl_4$ by citrate according to reported procedure.[S7] All glasswares were cleaned with aqua regia, and rinsed with ultrapure water (18.2 MΩ) before use. An aqueous solution of $HAuCl_4$ (1 mM, 100 mL) were brought to reflux while stirring. Once the gold solution was refluxing vigorously, an aqueous solution of sodium citrate (39 mM, 10 mL) was added quickly. Upon the addition of the sodium citrate, the color of the solution turned ruby-red, indicating the formation of the gold nanoparticles. The solution was refluxed for additional 30 min with vigorous stirring. The suspension was allowed to cool to room temperature. The UV-vis spectra showed a sharp and characteristic plasmon absorption maximum at 520 nm. The size of the gold nanoparticles was also confirmed using transmission electron microscopy (TEM).

Preparation of heme@AuNP

Heme-modified gold nanoparticles (heme@AuNP) were prepared using Au–S chemistry. The pH of the solution containing the citrated-capped AuNP was adjusted to pH 11 with the dropwise addition of 1 M NaOH. To the prepared AuNP solution (c.a. 13 nM, pH 11, 80 mL) was added a solution of the heme dimer with disulfide linkage (2.3 mg, 1.6×10^{-6} mol) and lipoic acid (9.0 mg, 4.4×10^{-5} mol) in 1 mL of DMSO, and the solution was incubated at room temperature in the dark. The mixed solution was then centrifuged at 14000 rpm for 15 min. To remove excess heme and the lipoic acid, the supernatant was decanted and the remaining solid was re-dispersed in a NaOH solution (pH 11). This procedure was repeated five times to obtain purified heme@AuNP. The heme@AuNP solution was stored in the dark until next use.

Quantification of heme molecules on AuNP

The number of heme moieties on each AuNP was determined by UV-vis spectroscopic measurements. To quantify the heme groups, the AuNP having strong absorption was decomposed by the addition of cyanide, which can convert to gold-cyanide complex, $K[Au(CN)_4]$.[S8] The 30 μL solution of heme@AuNP were treated with 30 μL of 1 M KCN and the AuNP etching process was followed by a loss of the characteristic plasmon absorbance at around 520 nm. After the reaction was finished, the adsorption originated from bis-cyanide complex of the heme was measured to calculate the number of heme moieties on AuNP.

Preparation of protein@AuNP

To a solution of apo-cyt b_{562} (10 μM, c.a. 8 μL), poly-cyt b_{562} (330 μM, c.a. 8 μL), and apo-dimer-cyt b_{562} (10 μM, c.a. 8 μL) in 50 mM Tris-HCl buffer (pH 7.3) containing 50 mM NaCl was added c.a. 3 μM heme@AuNP in 2 μL of aqueous NaOH solution (pH 11) and the mixture was incubated for 12 h to give corresponding protein–AuNP conjugates, cyt b_{562}@AuNP, poly-cyt b_{562}@AuNP, and [apo-dimer-cyt b_{562}@AuNP]$_{ass}$.

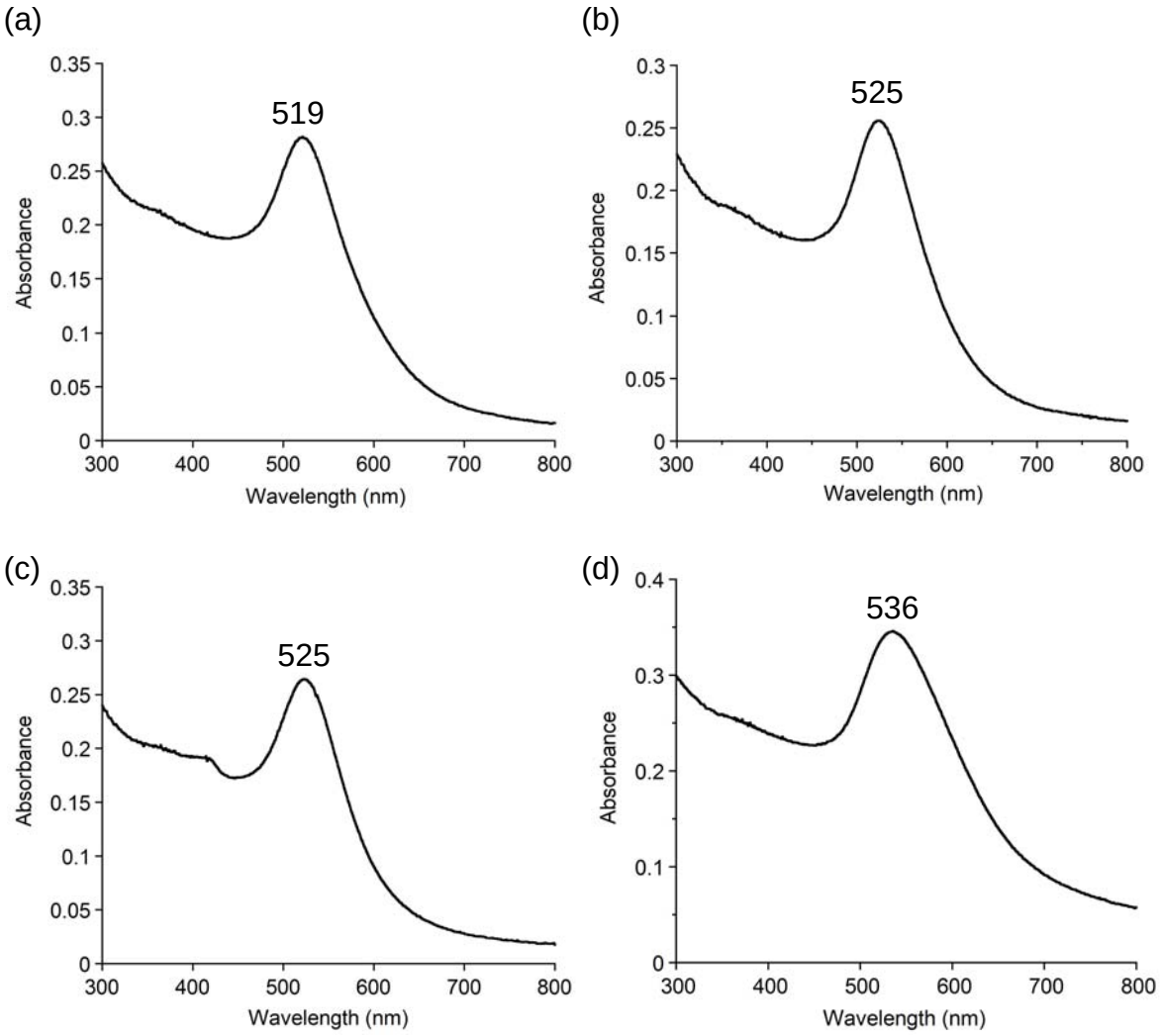


FIGURE S1. Absorption spectra of (a) heme@AuNP, (b) cyt b_{562}@AuNP, (c) poly-cyt b_{562}@AuNP, and (d) [apo-dimer-cyt b_{562}@AuNP]$_{ass}$ in Tris-HCl (pH 7.3) buffer.

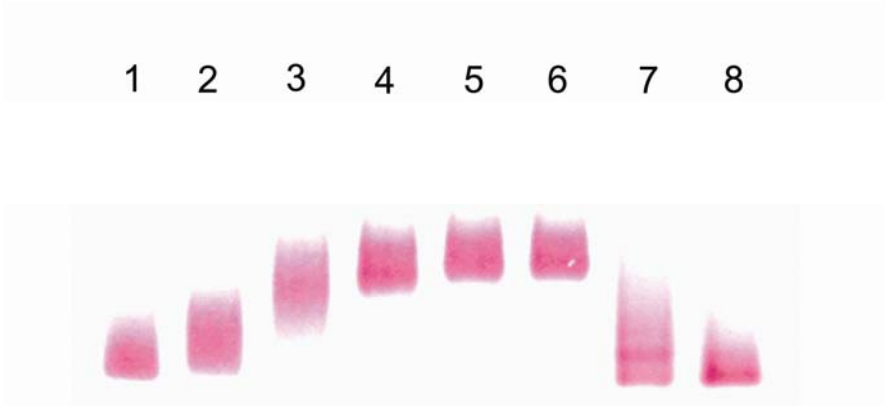


FIGURE S2. Binding analyses of heme@AuNP and apo-cyt b_{562} using agarose gel electrophoresis (1.5 % agarose, Tris/Borate/EDTA (TBE) buffer). The ratio of heme@AuNP: apo-cyt b_{562} =1:1 (Lane 1), 1:2 (Lane 2), 1:5 (Lane 3), 1:10 (Lane 4), 1:20 (Lane 5), 1:50 (Lane 6). Heme@AuNP (Lane 7) and AuNP protected with lipoic acid (Lane 8) were used as references.

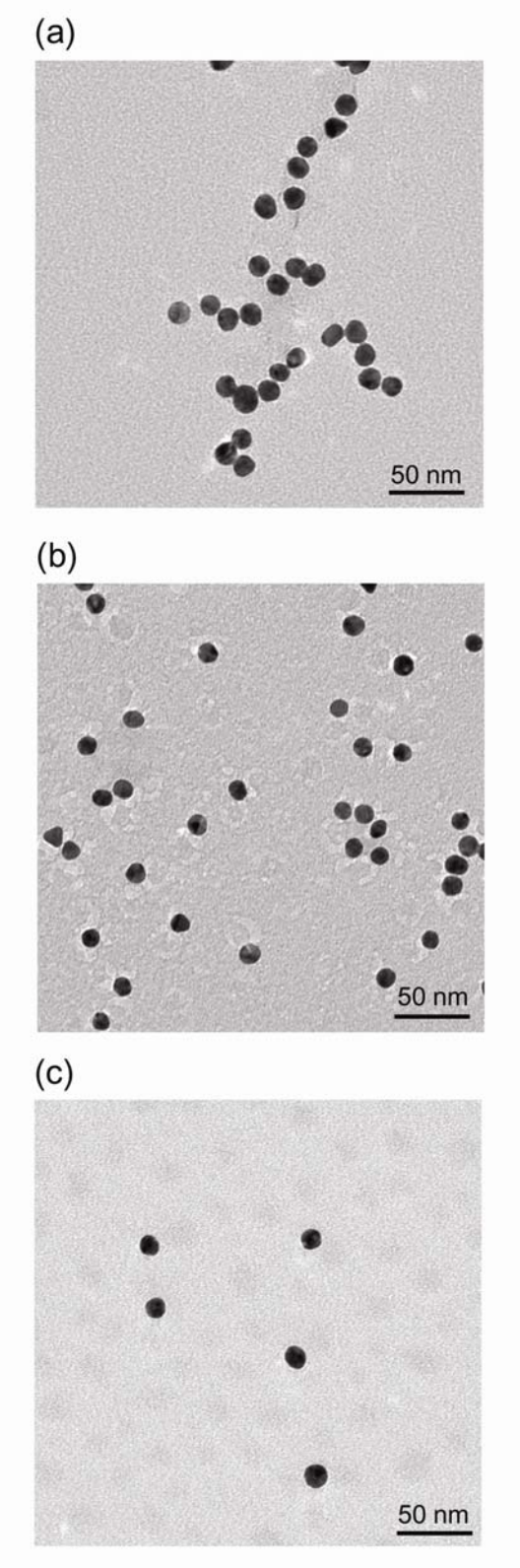


FIGURE S3. TEM images of (a) heme@AuNP, (b) cyt b_{562}@AuNP and (c) poly-cyt b_{562}@AuNP supported on carbon grids. Samples were prepared by drying of 5 μL of the protein solution on the grid, followed by washing with deionized water to remove buffer salts. Average diameter of the nanoparticles: heme@AuNP, 14.8 ± 0.86 nm; cyt b_{562}@AuNP, 13.8 ± 0.79 nm; poly-cyt b_{562}@AuNP, 14.7 ± 1.08 nm).

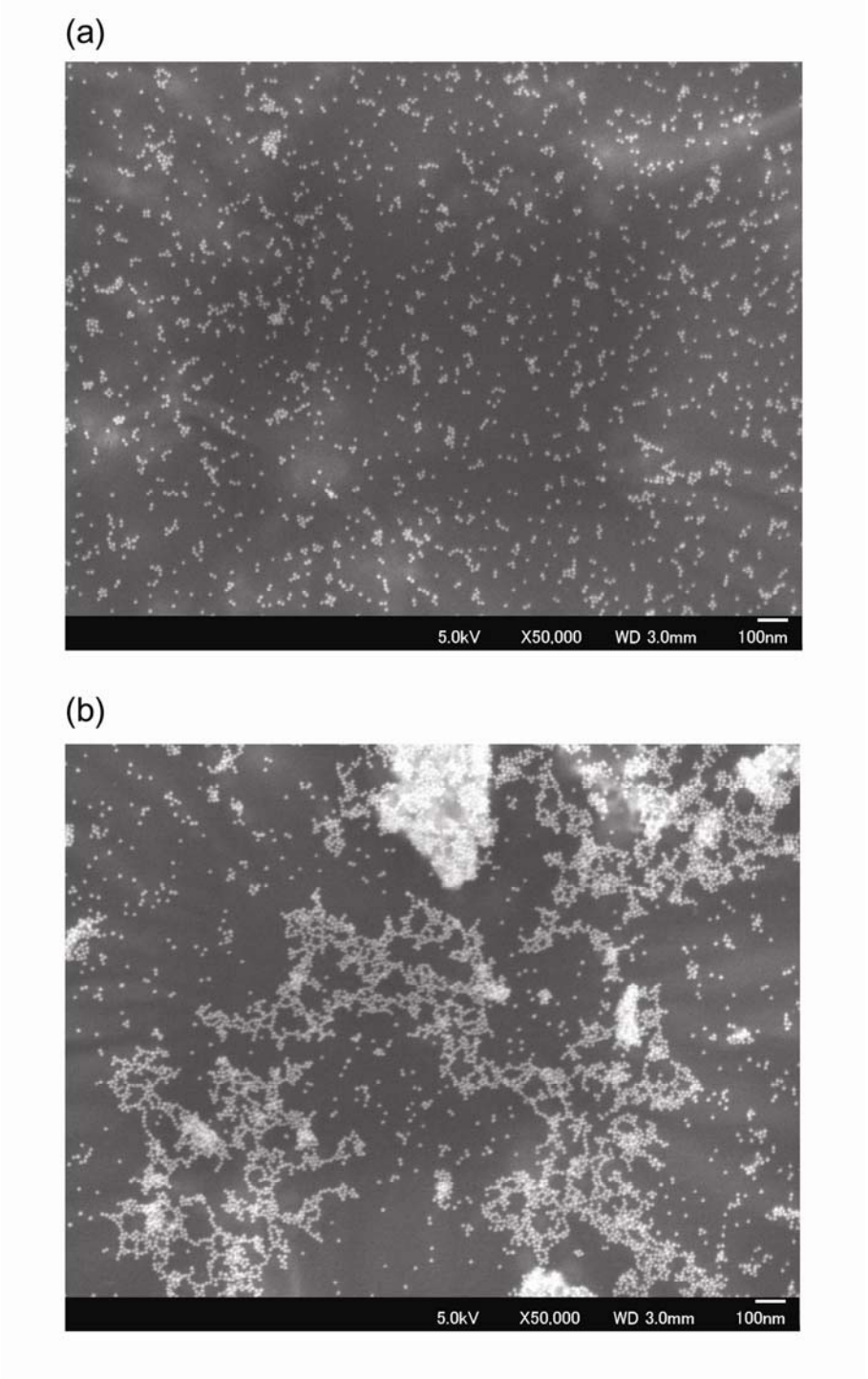


FIGURE S4. SEM images of (a) cyt b_{562}@AuNP and (b) [apo-dimer-cyt b_{562}@AuNP]$_{ass}$. The carbon grid with the samples prepared for the TEM measurements is fixed on the SEM sample folder using a conductive carbon adhesive tape.

Reference

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