Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Cofactor-specific Covalent Anchoring of Cytochrome b_{562} on Single-walled Carbon Nanotube by Click Chemistry

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Supporting information

Materials and Instruments

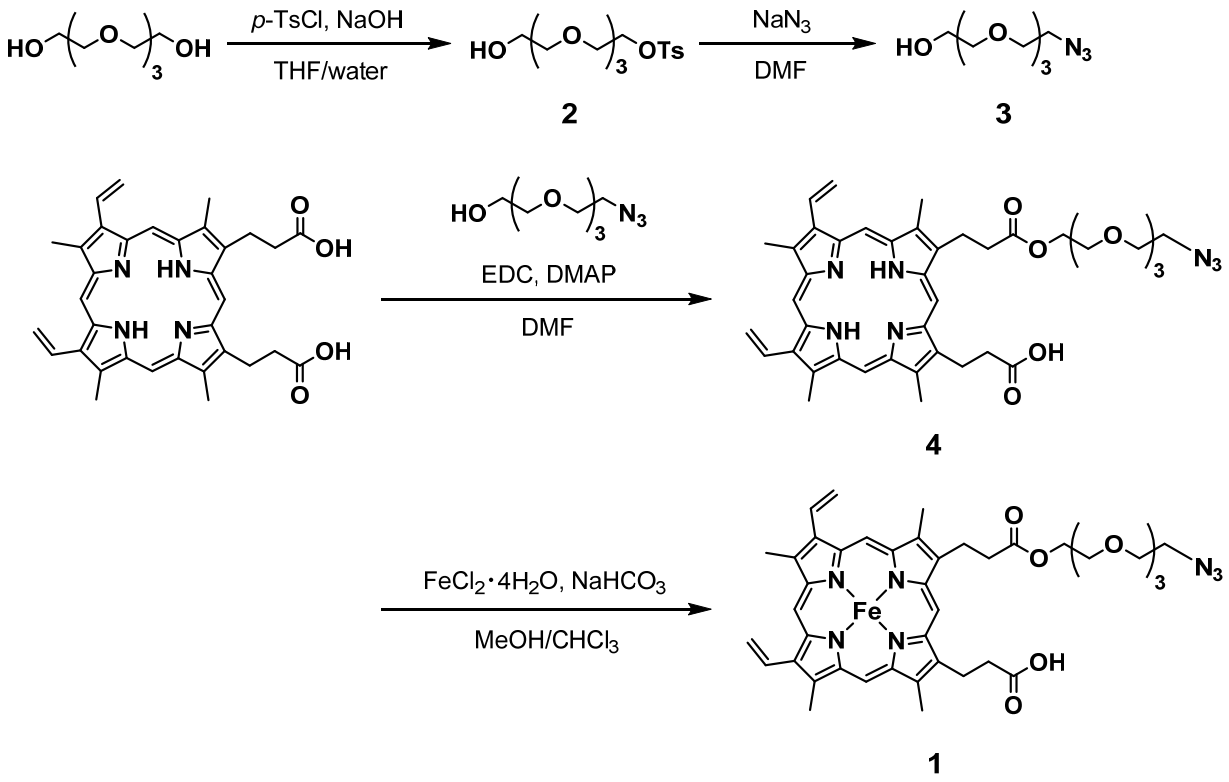
Instruments.

^{1}H NMR spectra (400 MHz) were recorded on a Bruker DPX400 NMR spectrometer, and chemical shifts were reported in ppm relative to the residual solvent resonance. ESI-TOF MS analyses were performed on a Bruker micrOTOF focus III mass spectrometer. FT-IR measurements were performed by a Jasco FT/IR-4000. UV-vis spectra were measured using a Shimadzu UV-3150 or UV-2550 spectrophotometer equipped with a thermostated cell holder. The pH values were monitored using an F-52 Horiba pH meter. Electrochemical measurements were conducted using a CompactStat potentiostat (Ivium Technologies) using a platinum mesh as a counter electrode and standard Ag|AgCl as a reference electrode. Atomic force microscopy (AFM) measurements were performed using an MFP-3DTM-SA microscope (Asylum Research). Raman Spectra were recorded on a Horiba-Jobin Yvon LabRAM ARAMIS spectrometer with excitation at 532 nm. The thermogravimetric analyses were performed with a TGA Q50 (TA Instruments) at 10 °C/min under N_2.

Materials.

Arc discharge SWNT was purchased from Carbon Solutions Inc. and purified as described previously.[S1] f-SWNT was synthesized according to the literature.[S1] Functionalization of the sidewall was confirmed by UV-vis spectroscopy and Raman spectroscopy (Fig. S1).[S2] Heme **1** and protoporphyrin IX derivatives **2**, **3**, and, **4** were synthesized as described previously with a slight modification.[S3] Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) was synthesized according to the literature.[S4] Other reagents and chemicals were purchased and used as received. An apo form of wild-type cytochrome b_{562} (apoCYT) was prepared according to a previous report with a slight modification.[S5]

Synthesis.



Scheme S1. Synthetic pathway of heme **1**

Alcohol 2. To a solution of tetraethylene glycol (11.92 g, 64.7 mmol) in THF (7 mL) was added dropwise a solution of NaOH (0.36g, 90.0 mmol) in THF (2 mL), and the reaction solution was cooled to 0 °C. After the solution was added dropwise *p*-TsCl (1.22 g, 6.4 mmol) in THF (3 mL) with vigorous stirring, the reaction mixture was stirred at 25 °C for 2 h. The solution was poured into iced water (25 mL), and the product was extracted with CH_2Cl_2 (10 mL x 3). The organic layer was washed with water (30 mL x 2) and brine (30 mL), and dried with Na_2SO_4. The solvent was removed in *vacuo* to give alcohol **2** as a pale yellow oil (1.86 g, 5.34 mmol, 83%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, 2H, J = 8.2 Hz), 7.34 (d, 2H, J = 8.2 Hz), 4.17 (t, 2H, J = 4.8 Hz), 3.70−3.59 (m, 14H), 2.45 (s, 3H) , 2.48 (br, 1H).

Alcohol 3. Alcohol **2** (1.86 g, 5.34 mmol) and sodium azide (1.38 g, 21.2 mmol) in dry DMF (20 mL) was stirred at 60 ºC for 5 h. After addition of water (10 mL), the product was extracted with EtOAc (15 mL x 3) and the organic layer was washed with water (10 mL) and brine (10 mL). The solvent was removed under reduced pressure to give alcohol **3** as a pale yellow oil (0.94 g, 4.29

mmol, 80%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.74−3.56 (m, 14H), 3.39 (t, 2H, 5.2 Hz), 2.45 (t, 1H, *J* = 6.0 Hz).

Porphyrin 4. To a solution of protoporphyrin IX (154 mg, 0.27 mmol) in anhydrous DMF (20 mL) was added a solution of EDC•HCl (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) (57.0 mg, 0.30 mmol) and DMAP (*N*,*N*-dimethyl-4-aminopyridine) (35.6 mg, 0.29 mmol) in DMF (5 mL) at 0 °C, followed by addition of a solution of alcohol **3** (90.8 mg, 0.41 mmol) in DMF (2 mL). The reaction solution was gradually warmed to room temperature, then stirred overnight. After addition of saturated NH_4Cl_{aq} (15 mL), the product was extracted with CH_2Cl_2 (30 mL x 4). The organic layer was washed with water (100 mL) and brine (80 mL), and dried with Na_2SO_4. After the solvent was removed in *vacuo*, the crude product was purified by silica gel chromatography (acetone/CH_2Cl_2 = 20−25% then MeOH/CH_2Cl_2 = 5%). The product was precipitated from hexane/CH_2Cl_2 to give porphyrin **4** as a brown solid (98 mg, 0.128 mmol, 47%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.24−10.1 (m, 4H), 8.30 (dd, 4H, *J* = 17.2, 11.2 Hz), 6.39 (d, 2H, *J* = 18.0 Hz), 6.20 (d, 2H, *J* = 11.2 Hz), 4.42−4.34 (m, 6H), 3.73−3.72 (m, 6H), 3.70−3.69 (m, 2H), 3.63 (t, 6H, *J* = 6.4 Hz), 3.49−3.32 (m, 10H), 3.25 (t, 2H, *J* = 7.2 Hz), 3.10 (t, 2H, *J* = 5.2 Hz), −3.9 (s, 2H); ESI-TOF MS (positive mode) *m*/*z* calcd. for $C_{42}H_{49}N_7O_7$ $[M + H]^+$ 764.37, found 764.34.

Heme 1. Porphyrin **4** (32.5 mg, 42.5 μmol), $FeCl_2 \cdot 4H_2O$ (174.5 mg, 878 μmol), and $NaHCO_3$ (11.0 mg, 131 μmol) in MeOH/$CHCl_3$ (1:2, 90 mL, N_2 bubbled) was stirred at 40 °C for 4 h. The solvent was evaporated to dryness, and the residue was purified by precipitation from hexane/CH_2Cl_2 to give heme **1** as a brown solid (30.5 mg, 37.3 μmol, 88%): FT-IR (KBr) *v* (cm^{-1}) 1734 (C=O), 2106 (−N=N=N). ESI-TOF MS (positive mode) *m*/*z* calcd. for $C_{42}H_{47}N_7O_7Fe$ $[M]^+$ 817.29, found 817.29).

Incorporation of heme 1 into apoCYT.

Heme **1** (0.12 mg, 150 nmol) in pyridine (50 μL) was added dropwise into a solution of apoCYT (114 μM) in 2 mL of 100 mM MOPS buffer (pH 7.0) with gentle shaking on ice. The reaction mixture was stirred overnight at 4 °C, then concentrated by ultrafiltration. CYT•**1** was purified using a DEAE Sepharose Fast Flow column (2 x 5 cm, GE Healthcare) pre-equilibrated using 0.1 M MOPS buffer (pH 7.0) to remove the excess cofactor. The collected fractions were concentrated and stored in the dark at −80 °C. A solution of CYT•**1** (5.5 μM, 600 μL) in MOPS buffer was mixed with 20 μL of 20 mM sodium ascorbate in 100 mM MOPS buffer (pH 7.0) to prepare the reduced form of CYT•**1**.

CuAAC reaction between CYT•1 and 5-hexynoic acid.

5-Hexynoic acid (0.11 mg, 1.0 μmol) in DMSO (1.0 μL) was diluted with 2 wt% sodium cholate solution dissolved in 317 μL of 100 mM MOPS buffer (pH 7.0). CYT•**1** (35 μM, 5.25 nmol) in buffer (150 μL), $CuSO_4$ (20 mM, 50 nmol) in water (2.5 μL), THPTA (50 mM, 250 nmol) in water (5.0 μL), and sodium ascorbate (100 mM, 2.5 μmol) in water (25 μL) were added to the solution. The reaction mixture was incubated for 15 h at 4 °C. After the reaction, potassium ferricyanide (100

mM, 10 μmol) in water (100 μL) was added and the solution was incubated for a few minutes and then concentrated by ultrafiltration to remove ferricyanide. The product was characterized by ESI-TOF MS (positive mode) (*m*/*z* calcd. for $C_{825}H_{1303}N_{217}O_{247}S_1$ $[M + 7\ H^+]^{7+}$ 1816.59; found 1816.63).

Preparation of SWNT−CYT conjugate.

To a suspension of f-SWNT (0.1 mg) in NMP (10 mL) at 0 °C was added a solution of tetrabutylammonium fluoride (1 M in THF) (15 μL). The reaction mixture was stirred at room temperature for 1 h. After filtration on a PTFE membrane (0.2 μm), the f-SWNT was washed with water, NMP, THF, CH_2Cl_2 and Et_2O, then dispersed in an aqueous solution (5 mL) containing 2 wt% sodium cholate. The suspension was centrifuged (3000 rpm, 10 min) to remove precipitates including bundles and then 95% of supernatant was used for in the subsequent reaction. A solution (18 μL) of CYT•**1** (1.8 nmol) in 50 mM MOPS buffer (pH 7.0), sodium ascorbate (5.94 mM in MOPS buffer) (10 μL, 59.4 nmol), $CuSO_4$ (3.94 mM in MOPS buffer) (2 μL, 7.9 nmol), and THPTA (spatula tip) were added to the dispersion of f-SWNT. The reaction solution was gently mixed at 4 ºC for 2 days and then dialyzed against MOPS buffer (50 mM, pH 7.0) to remove the surfactant, copper catalyst and ascorbate. After concentration by ultrafiltration, the dispersion of the conjugate was stored at 4 °C.

AFM measurements.

The conjugate dispersed in 3 μL of MOPS buffer was dropped on to a freshly-cleaved mica substrate. After incubation for a few minutes, the dispersion was removed from the substrate, and the surface of the substrate was rinsed well with water. The surface of the mica substrate was dried under flowing N_2 for 1 min prior to the measurement. The sample was measured by tapping mode AFM.

Electrochemical measurements.

The hybrid dispersion (1 mL) was centrifuged (10000 rpm, 10 min), and the precipitate was collected, and then re-dispersed in 5 mL of MOPS buffer (50 mM, pH 7.0). This step was repeated three times to remove excess or non-covalently absorbed proteins. Finally, the sample was dispersed in 10 μL MOPS buffer. The resultant dispersion was mixed with 10 μL Nafion® solution (5 wt%), and 5 μL of the mixture was casted on the polished GC electrode and dried under nitrogen. The modified GC electrode was rinsed with water and transferred to an electrochemical cell containing 50 mM MOPS buffer (pH 7.0). The differential pulse voltammograms were obtained in the potential range −0.6 to +0.1 V (vs. Ag|AgCl reference electrode) at a scan rate of 10 mVs^{-1}.

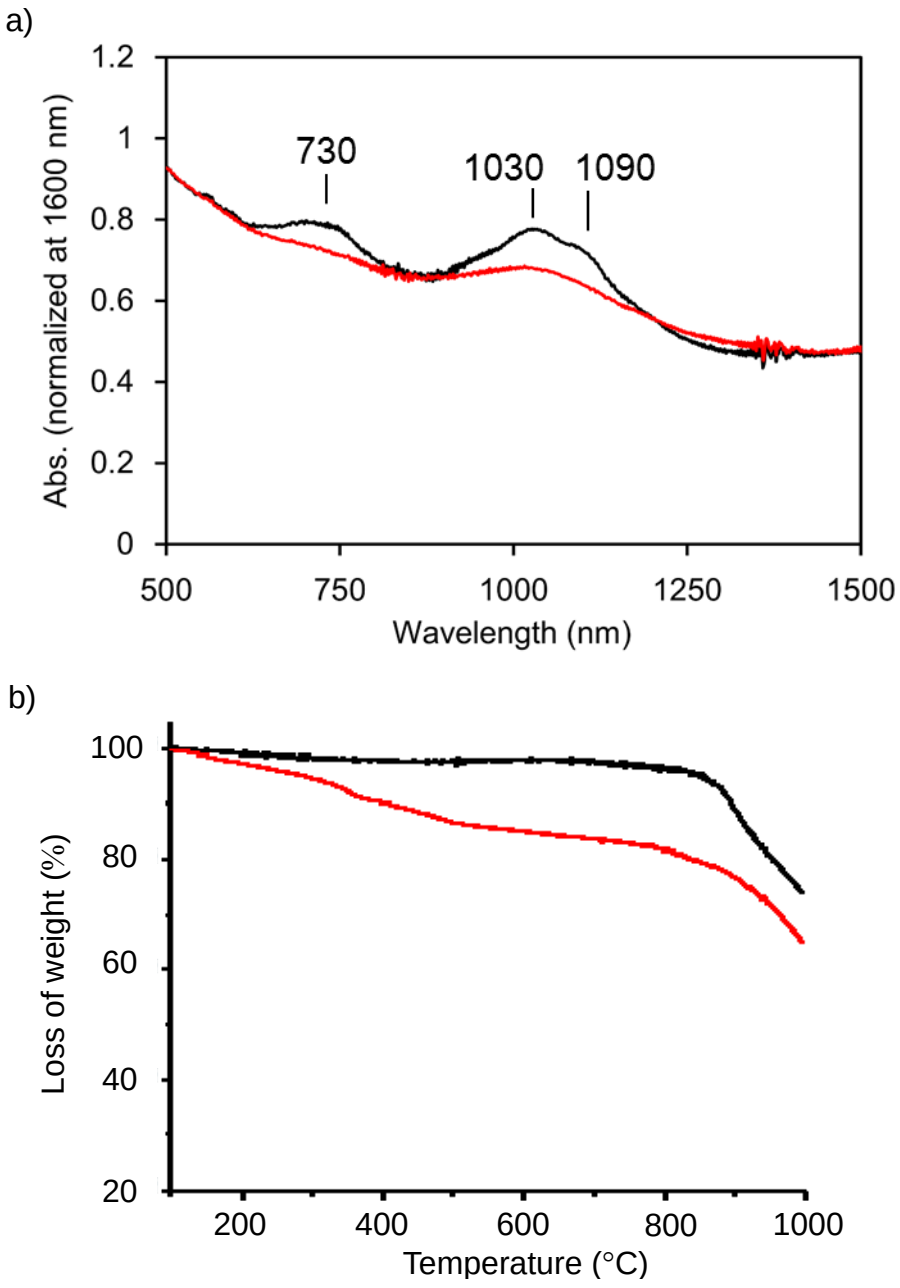


Fig. S1 UV-vis spectra of p-SWNT (black) and f-SWNT (red) dispersed in NMP (0.25 mg/mL). These data were normalized at 1600 nm. (b) Thermogravimetric analyses of purified SWNT (black) and f-SWNT (red) between 100 and 600 °C. The weight loss corresponding to the functionalization of the 4-ethynylphenyl group in f-SWNT is about 13%, suggesting that approximately one 4-ethynylphenyl group is attached per 100 carbon atoms of the f-SWNT sidewall.

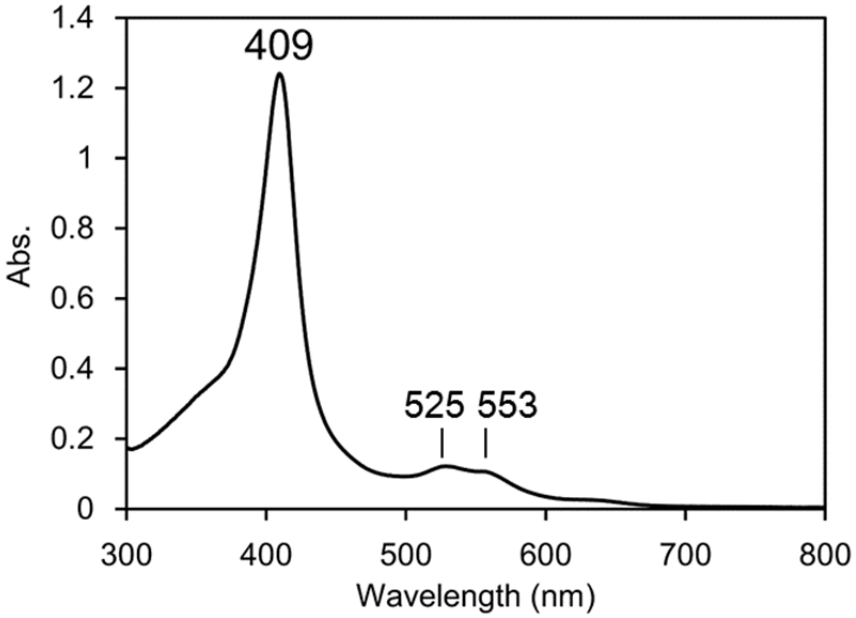


Fig. S2 UV-vis spectrum of heme **1** in pyridine.

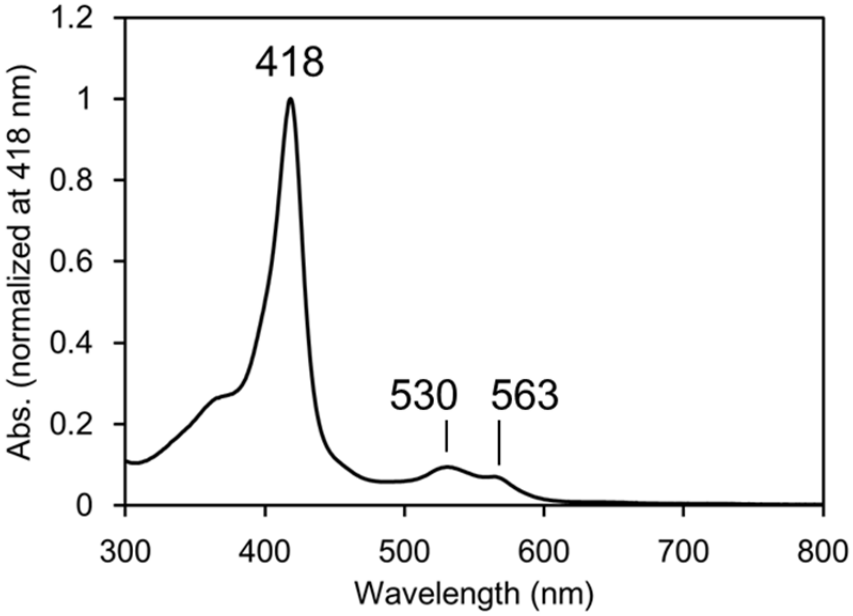


Fig. S3 UV-vis spectrum of CYT•**1** (75.4 nmol) in 100 mM MOPS buffer (pH 7.0) with sodium cholate (2 wt%, 2 mL). The spectrum was measured after incubation for 3 days at 4 ºC.

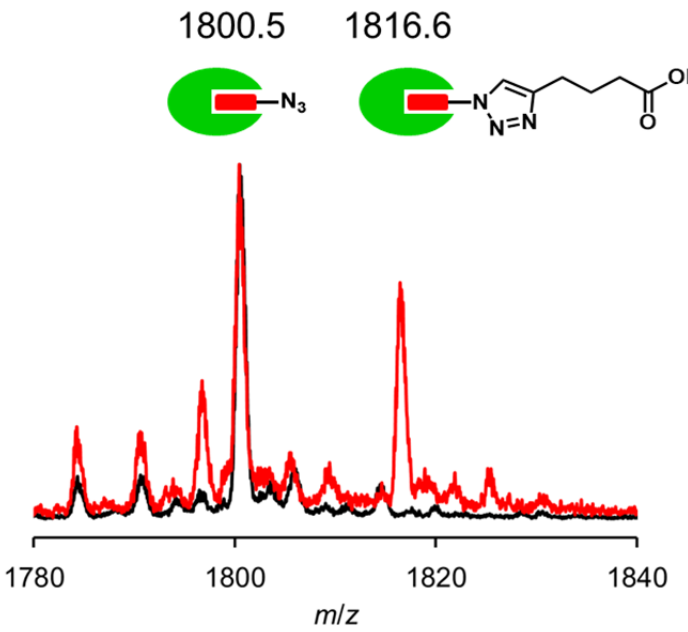


Fig. S4 ESI-TOF-MS of CYT•**1** (black) and the product after the CuAAC reaction between CYT•**1** and 5-hexynoic acid in the presence of sodium cholate.

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