Supplementary Information

Synthesis and characterisation of uniform CoPt nanoparticles using red blood cell ghosts conjugated with peptides on their inner surface

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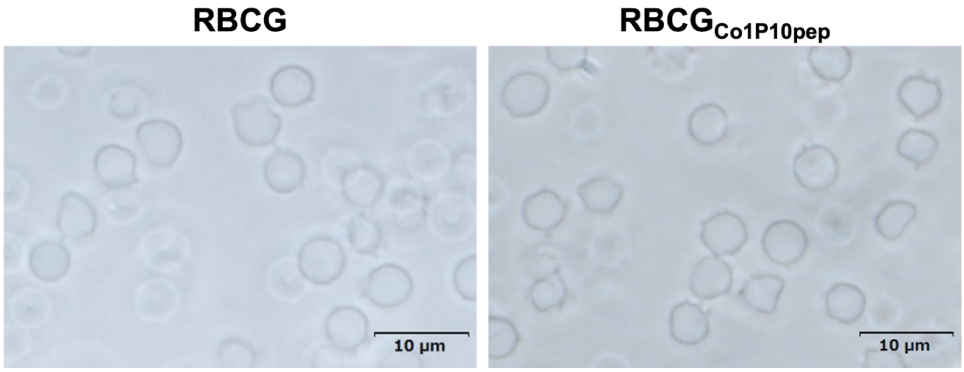

Fig. S1 Phase contrast microscopy images of RBCG and **RBCG$_{Co1P10pep}$**.

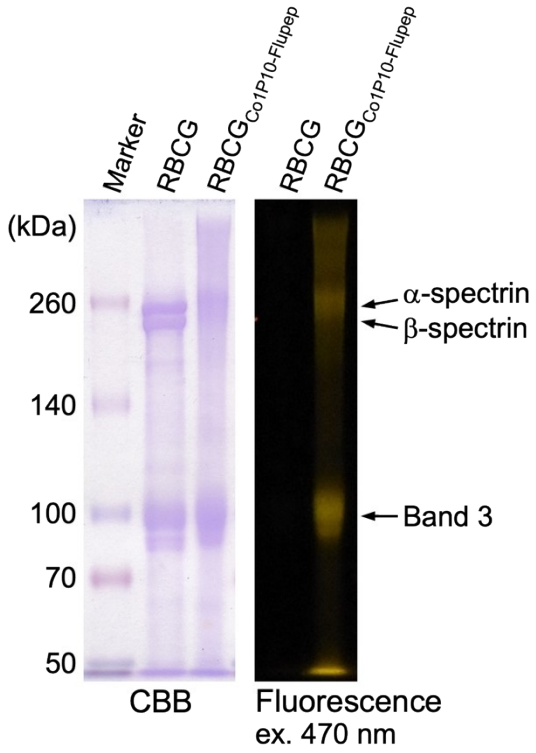

Fig. S2 6% SDS-PAGE images of RBCG and **RBCG$_{Co1P10-Flupep}$**, shown in fluorescence and after staining with Coomassie dye.

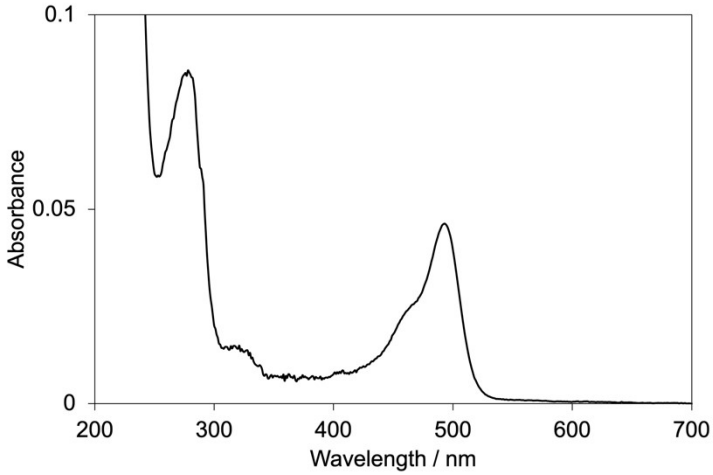

Fig. S3 UV−vis spectrum of **RBCG$_{Co1P10\text{-}Flupep}$** treated with 1% SDS (protein concentration 50 μg/mL).

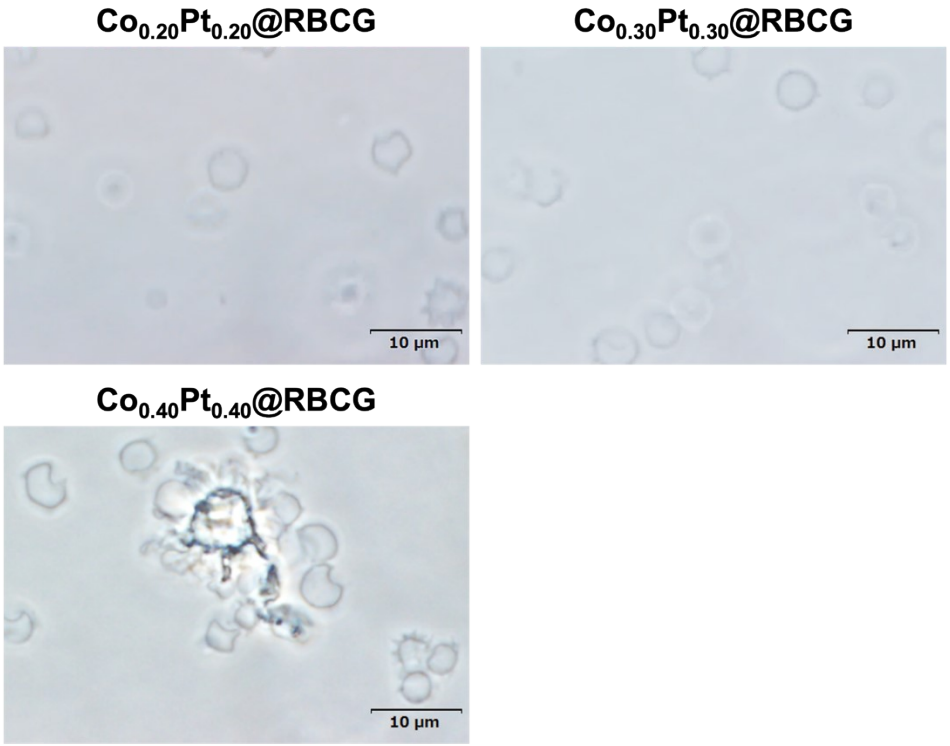

Fig. S4 Phase contrast microscopy images of **$Co_{0.20}Pt_{0.20}$@RBCG**, **$Co_{0.30}Pt_{0.30}$@RBCG**, and **$Co_{0.40}Pt_{0.40}$@RBCG**.

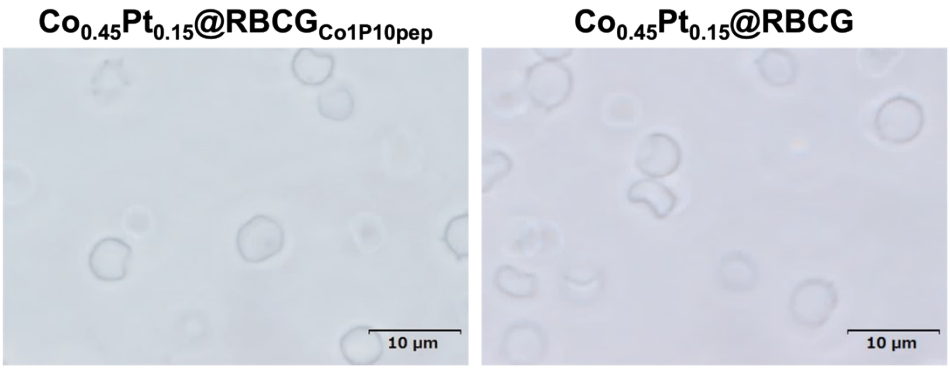

Fig. S5 Phase contrast microscopy images of **$Co_{0.45}Pt_{0.15}@RBCG_{Co1P10pep}$** and **$Co_{0.45}Pt_{0.15}@RBCG$**.

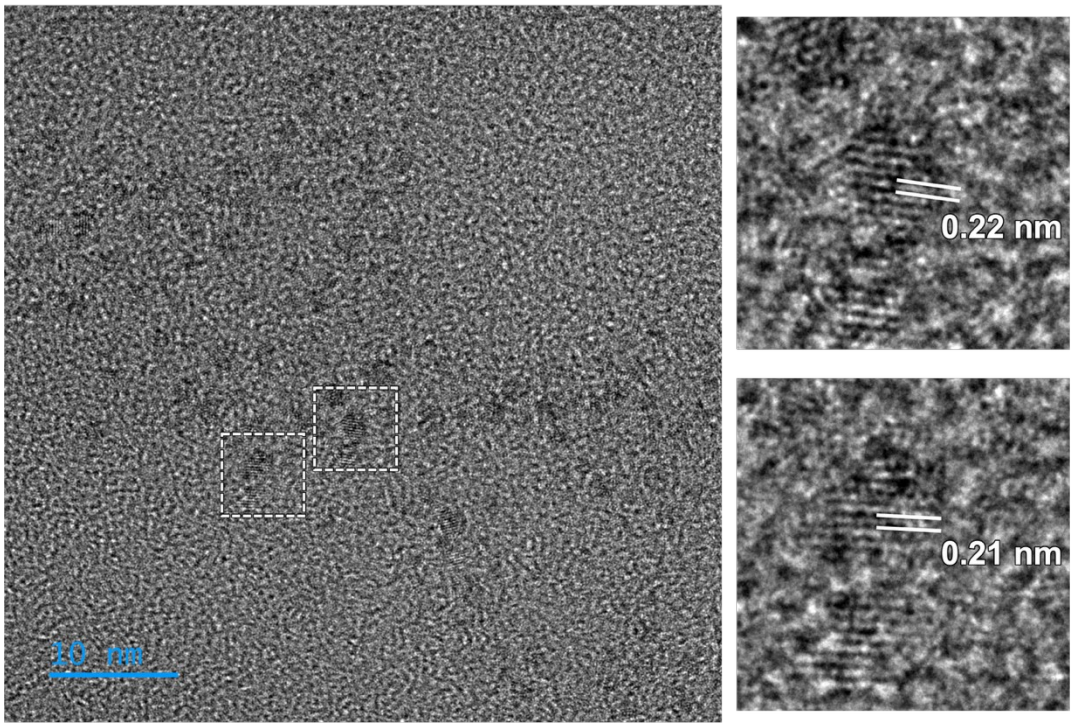

Fig. S6 TEM images of CoPt NPs of **$Co_{0.45}Pt_{0.15}@RBCG_{Co1P10pep}$**. The images on the right are enlarged views of the area enclosed by the dotted line, and NPs show the lattice fringes.

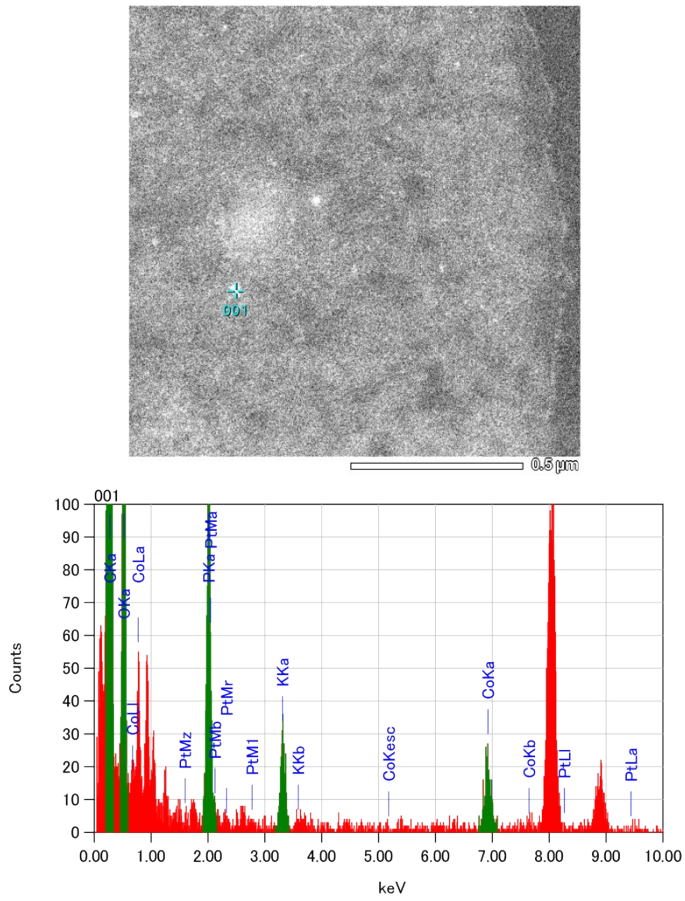

Fig. S7 TEM image and EDS point analysis of a nanoparticle in **$Co_{0.45}Pt_{0.15}$@RBCG**.

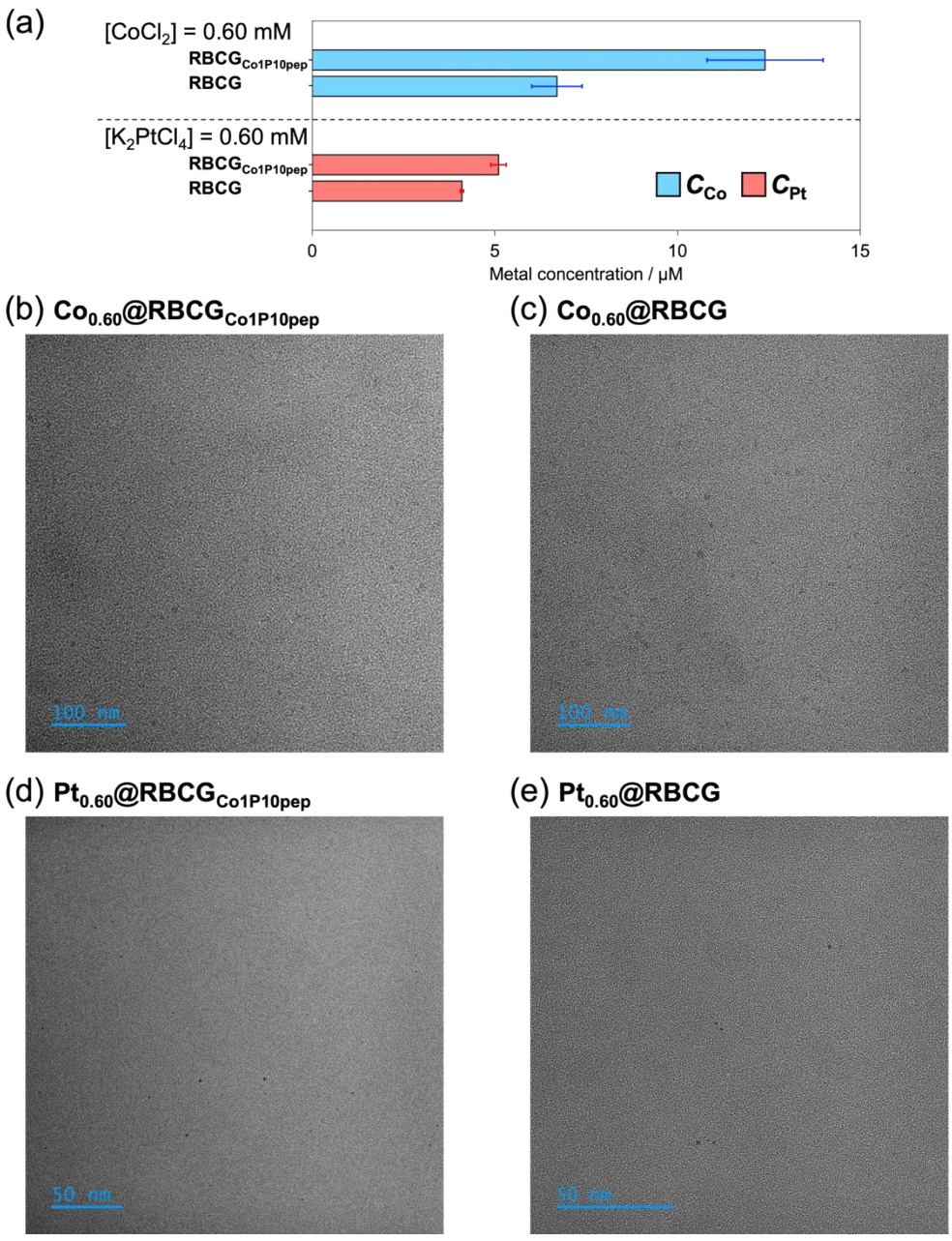

Fig. S8 (a) Bar graph summarizing Co and Pt concentrations (C_{Co} and C_{Pt}) per 100 μg/mL protein concentration for **$Co_{0.60}$@RBCG$_{Co1P10pep}$**, **$Co_{0.60}$@RBCG**, **$Pt_{0.60}$@RBCG$_{Co1P10pep}$** and **$Pt_{0.60}$@RBCG**. Each value is the average of at least three experimental repeats. Error bars represent the mean ± standard deviation of C_{Co} (blue) and C_{Pt} (red). The C_{Pt} values of **$Co_{0.60}$@RBCG$_{Co1P10pep}$** and **$Co_{0.60}$@RBCG** were 0 μM. The C_{Co} values of **$Pt_{0.60}$@RBCG$_{Co1P10pep}$** and **$Pt_{0.60}$@RBCG** were 0 μM. TEM images of (b) **$Co_{0.60}$@RBCG$_{Co1P10pep}$**, (c) **$Co_{0.60}$@RBCG**, (d) **$Pt_{0.60}$@RBCG$_{Co1P10pep}$**, and (e)

$Pt_{0.60}$@RBCG.

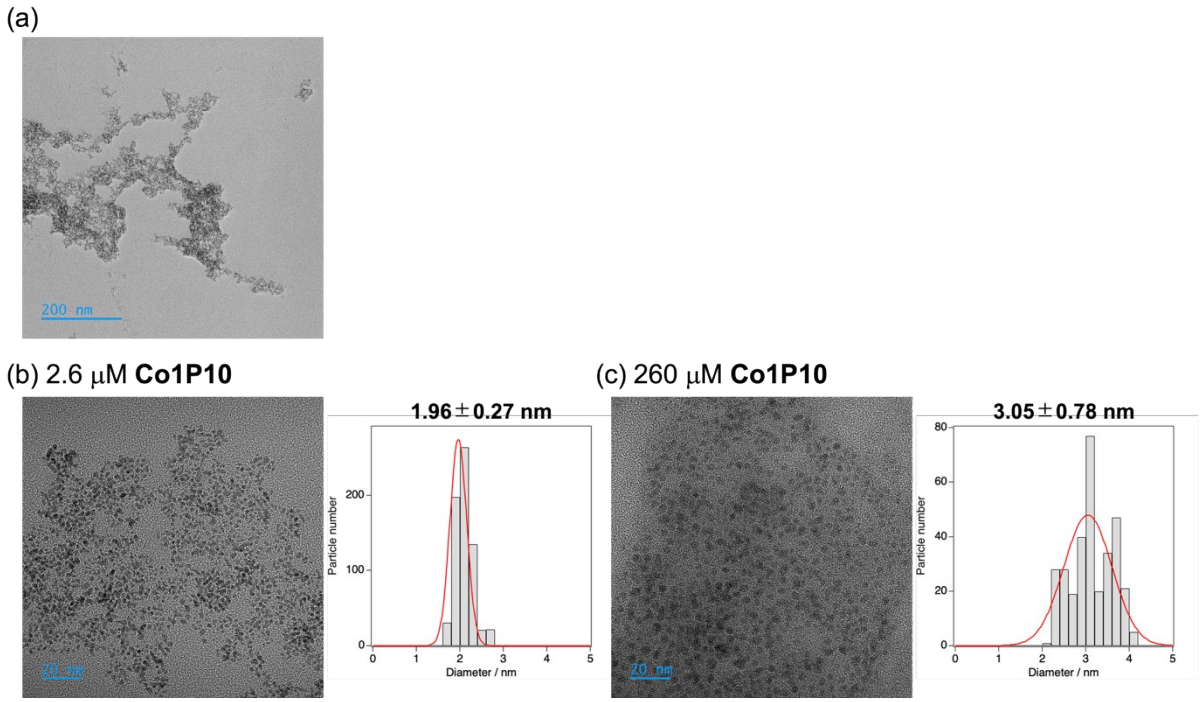

Fig. S9 (a) TEM image of CoPt NPs synthesized in the absence of **Co1P10** and RBCG. TEM images (left) and size distribution histograms (right) of CoPt NPs synthesized without RBCG in the presence of (a) 2.6 μM **Co1P10** and (a) 260 μM **Co1P10**. CoPt NPs were synthesized with $CoCl_2$/K_2PtCl_4 concentrations of 0.45/0.15 mM. The error indicates the standard deviation.

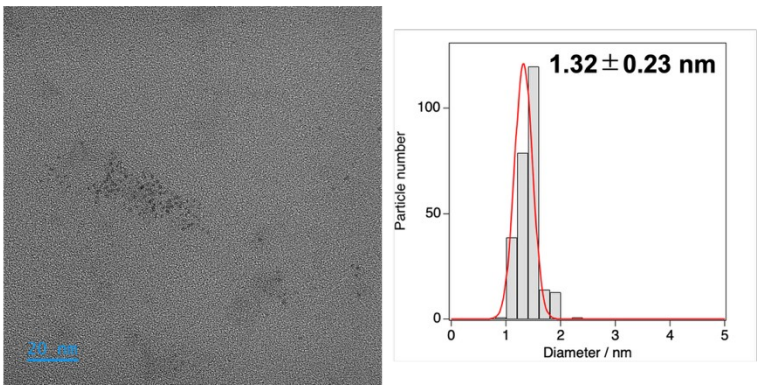

Fig. S10 TEM image (left) and size distribution histogram (right) of CoPt NPs of **$Co_{0.45}Pt_{0.15}$@$RBCG_{Co1P10pep(reduced)}$**. The error indicates the standard deviation.

Materials

Reagents were purchased from Watanabe Chemical Ind., Ltd., FUJIFILM Wako Pure Chemical Co., Tokyo Chemical Industry Co., Nacalai Tesque, and Sigma-Aldrich and used without further purification. Preserved horse blood was purchased from Japan Bio Serum Co., Ltd.

Physical Measurements

High-performance liquid chromatography (HPLC) was performed using a JASCO liquid chromatography system. MALDI-TOF mass spectra were acquired using a Shimadzu AXIMA with α-cyano-4-hydroxycinnamic acid as a matrix. The Co and Pt concentrations in **CoPt@RBCG** samples were determined using inductively coupled plasma optical emission spectrometry (ICPS-8100CL, Shimadzu, Co. Ltd.). The UV−vis absorption spectra were measured using a JASCO V-630 spectrophotometre. Phase-contrast and fluorescence microscopy measurements were performed using an IX73P1F microscope (Olympus). Transmission electron microscope images were obtained using a JEOL JEM-ARM200F microscope (200 kV). The sample solution was applied to a copper grid covered with a thin amorphous carbon film. The magnetic measurements were performed using a SQUID magnetometer MPMS XL7AC (Quantum-Design).

Synthesis of cys-Co1P10 peptide

Cys-**Co1P10** peptide, H-Cys(Trt)-Gly-Phe(4-CN)-Gly-His(Trt)-Tyr(tBu)-Pro-Thr(tBu)-Leu-Pro-Leu-Gly-Ser(tBu)-Ser(tBu)-Thr(tBu)-Tyr(tBu)-alko Resin was synthesised on Fmoc-Tyr(tBu)-alko Resin (Watanabe Chemical Ind. Ltd.) using standard Fmoc-based solid-phase chemistry (4 equiv. Fmoc-amino acids). *N,N*-Dimethylformamide (DMF) solutions of *N*-methylmorphiline (NMM, 0.4 M) and 1-[bis (dimethylamino)methylene]-5-chloro-1*H*-

benzotriazolium 3-oxide hexafluorophosphate (HCTU, 4 equiv.) or 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HBTU, 4 equiv.) were used as the coupling reagents. Each condensation reaction was conducted at RT for at least 20 min. The Fmoc groups were deprotected from the resin using 20% piperidine in DMF. The peptidyl resin was then washed with DMF and dried under vacuum. The peptide was deprotected and cleaved from the resin by treatment with a cleavage cocktail (*m*-cresol / thioanisole / trifluoroaceticacid (TFA) / 1,2-ethanedithio = 2:12:80:6 v/v/v/v). The mixture was incubated at RT for 2 h. After filtration, the peptides were precipitated by adding ice-cold diethyl ether. After centrifugation, the peptides were washed thrice with diethyl ether. The precipitated peptides were dried under vacuum. The crude product was purified by RP-HPLC with water/acetonitrile (both containing 0.1% TFA, 80:20 to 72.5:27.5 v/v for 45 min, linear gradient, 3 mL/min, detected at 220 nm). MALDI-TOF-MS: *m/z* found 1724.6 ($[M+H]^+$), calculated 1724.8, found 1746.6 ($[M+Na]^+$), calcd. 1746.8, and found 1762.5 ($[M+K]^+$), calcd. 1762.9 (Fig. S11).

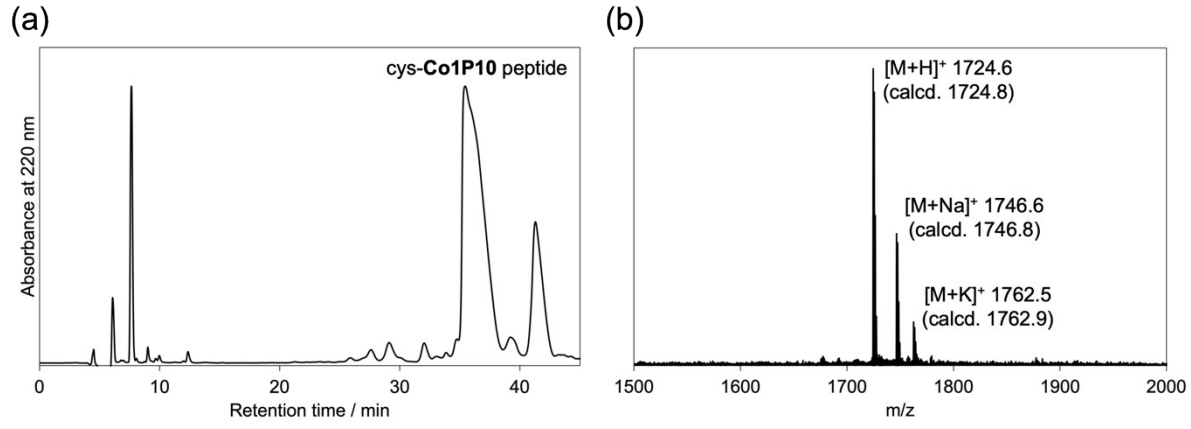

Fig. S11 (a) HPLC chromatogram of crude cys-**Co1P10** peptide. The eluted peak at 36 min was collected. (b) MALDI-TOF mass spectrum of the purified cys-**Co1P10** peptide.

Synthesis of Ac-cys-Co1P10-K (Ac-CGF$_{CN}$GHYPTLPLGSSTYK)

Ac-cys-**Co1P10**-K, Ac-Cys(Trt)-Gly-Phe(4-CN)-Gly-His(Trt)-Tyr(tBu)-Pro-Thr(tBu)-Leu-Pro-Leu-Gly-Ser(tBu)-Ser(tBu)-Thr(tBu)-Tyr(tBu)-Lys-OH was prepared by the procedure described above using Fmoc-Lys(OtBu)-Wang resin. *N*-acetylated peptides were obtained via on-resin acetylation with acetic anhydride in DMF. The crude product was purified by RP-HPLC with water/acetonitrile (both containing 0.1% TFA, 80:20 to 70:30 v/v for 40 min, linear gradient: 3 mL/min, detected at 220 nm). MALDI-TOF-MS: *m/z* found 1895.4 ([M+H]$^+$) calcd. 1895.1 (Fig. S12).

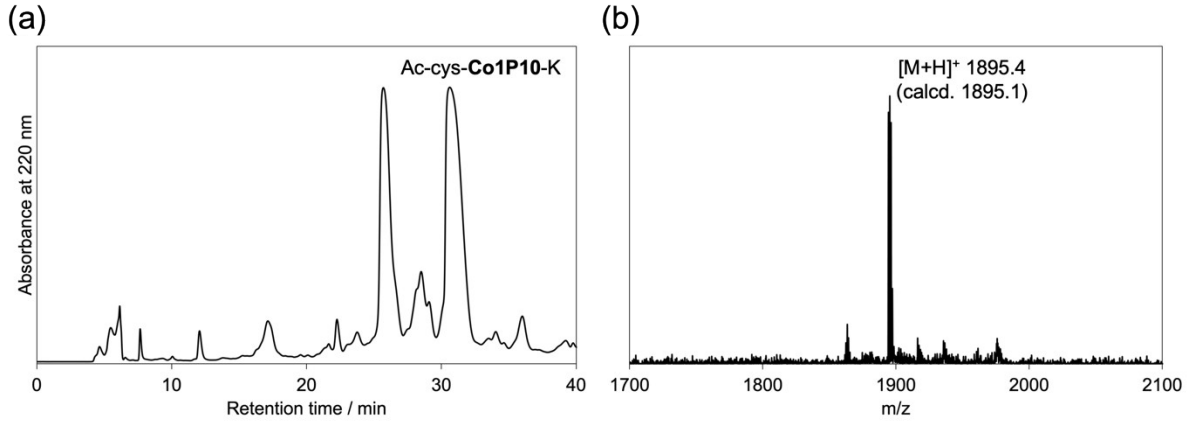

Fig. S12 (a) HPLC chromatogram of crude Ac-cys-**Co1P10**-K. The eluted peak at 31 min was collected. (b) MALDI-TOF mass spectrum of the purified Ac-cys-**Co1P10**-K.

Synthesis of cys-Co1P10-Flu peptide

A DMSO solution of NHS-Fluorescein (1.2 equiv.) was added to 2 mM Ac-cys-**Co1P10**-K in 0.1 M $NaHCO_3$. The reaction mixture was stirred at 25 °C for 20 h in the dark. The unreacted fluorescein was removed using a C18 Spin Column (MonoSpin C18; CL Sciences). The resulting peptide solution was purified using RP-HPLC with water/acetonitrile (both containing 0.1% TFA, 70:30 to 65:35 v/v for 60 min, 1 mL/min, detected at 220 nm).

MALDI-TOF-MS: *m/z* found 2253.1 ([M+H]$^+$), calcd. 2253.4, found 2275.0 ([M+Na]$^+$), calcd. 2275.4, and found 2290.9 ([M+K]$^+$), calcd. 2291.5 (Fig. S13).

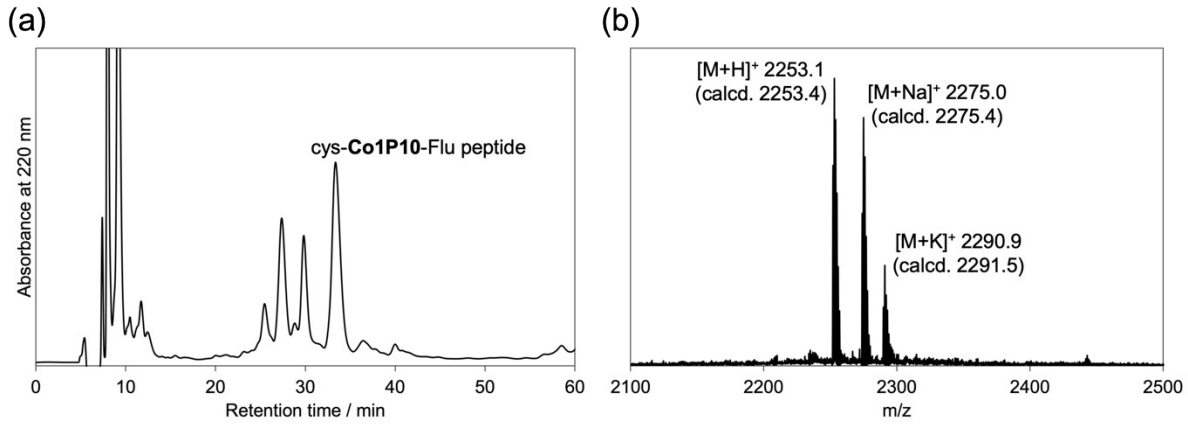

Fig. S13 (a) HPLC chromatogram of crude cys-**Co1P10**-Flu peptide. The eluted peak at 33 min was collected. (b) MALDI-TOF mass spectrum of the purified cys-**Co1P10**-Flu peptide.

Synthesis of Co1P10 (HYPTLPLGSSTY)

Co1P10, H-His(Trt)-Tyr(tBu)-Pro-Thr(tBu)-Leu-Pro-Leu-Gly-Ser(tBu)-Ser(tBu)-Thr(tBu)-Tyr(tBu)-alko Resin was synthesised using the same procedure as cys-**Co1P10** peptide described above. The crude product was purified by RP-HPLC with water/acetonitrile (both containing 0.1% TFA, 80.5:19.5 to 76.5:23.5 v/v for 48 min, linear gradient: 3 mL/min, detected at 220 nm). MALDI-TOF-MS: *m/z* found 1336.4 ([M+H]$^+$), calcd. 1336.4, found 1358.5 ([M+Na]$^+$), calcd. 1358.3, found 1374.4 ([M+K]$^+$), calcd. 1374.4, and found 1380.7 ([M+2Na-H]$^+$), calcd. 1380.2 (Fig. S14).

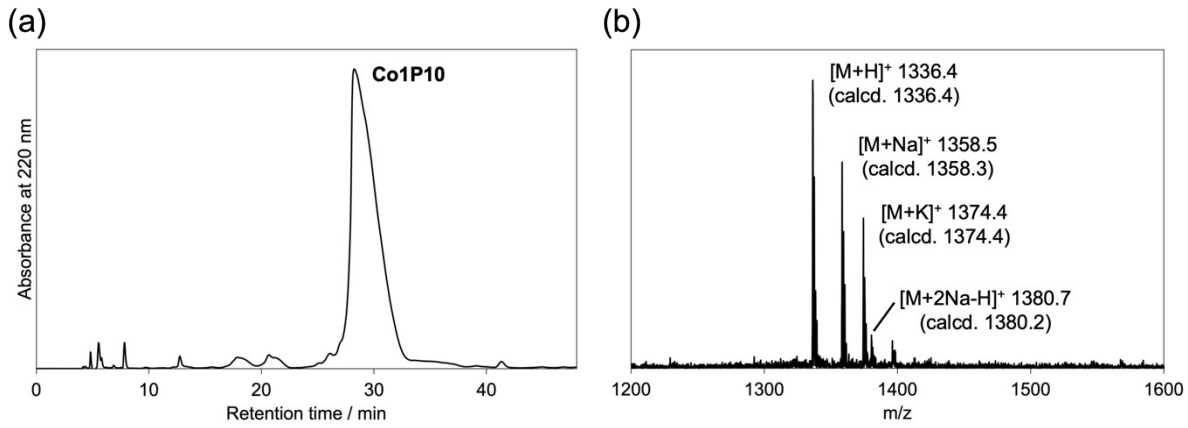

Fig. S14 (a) HPLC chromatogram of crude **Co1P10**. The eluted peak at 28 min was collected. (b) MALDI-TOF mass spectrum of the purified **Co1P10**.

Modification of RBCG with Co1P10 peptide (RBCG$_{Co1P10pep}$)

RBCGs were prepared by hypotonic lysis of RBCs in phosphate buffer, according to previously reported procedures.[1–3] The final RBCG pellet was resuspended in 5P7.5 buffer (5 mM sodium phosphate, 10 mM KCl, and 50 μM $MgCl_2$•$6H_2O$, pH 7.5). Total protein concentrations were assayed using bicinchoninic acid assay reagent with bovine serum albumin as the standard. A buffer solution of *N*-(4-maleimidobutyryloxy) sulfosuccinimide and sodium salt (sulfo-GMBS) was added to the RBCG suspension in 5P7.5 buffer (protein concentration of 400 μg/mL, [sulfo-GMBS] = 0.5 mM). The reaction mixture was stirred at 4 °C for 2 h and centrifuged to harvest maleimide-modified RBCGs. The maleimide-modified RBCGs were washed seven times with 5P7.5 buffer and resuspended in TCEP buffer (530 μM TCEP, 5 mM Na_2HPO_4, 10 mM KCl, 50 μM $MgCl_2$). A buffer solution of the cys-**Co1P10** peptide was added to a suspension of maleimide-modified RBCGs ([peptide] = 0.30 mM). The reaction mixture was stirred at 4 °C for 18 h and centrifuged to harvest **RBCG$_{Co1P10pep}$**. **RBCG$_{Co1P10pep}$** was washed seven times with the 5P7.5 buffer.

Modification of RBCG with fluorescein-labeled Co1P10 peptide ($RBCG_{Co1P10\text{-}Flupep}$)

Modification of RBCG with fluorescein-labeled **Co1P10** peptide was performed according to the same procedure as modification of RBCG with **Co1P10** peptide, except that cys-**Co1P10**-Flu peptide was used instead of cys-**Co1P10** peptide.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE samples were prepared by mixing 30 μL of RBCG suspension with 30 μL of lysis buffer (0.1 M Tris-HCl, 0.004% BPB, 40% glycerin, 2% SDS) and heating at 80 °C for 30 min. The samples were separated using 6% SDS-PAGE. After electrophoresis, a fluorescent image was captured under an LED transilluminator LED470-DF36w (MeCan Imaging Inc., Japan). Proteins in the gel were stained with the CBB-R250 dye.

Calculation of approximate number of fluorescein-labeled Co1P10 peptide per RBCG

The approximate number of fluorescein-labelled **Co1P10** peptides per RBCG (N_{pep}) was calculated using the following formula:

$$N_{pep} = \frac{\text{Peptide concentration} \times 6.02 \times 10^{23} \times 1 \times 10^{-3}}{\text{cell density} \times \frac{50}{12}},$$

where "Peptide concentration" is the concentration (in units of mol/L) per 50 μg/mL protein concentration, and "cell density" is the number of RBCGs per mL (protein concentration 12 μg/mL). The peptide concentration was determined from the absorbance of fluorescein (molar extinction coefficient, 73,500 $cm^{-1}M^{-1}$ at 493 nm) (Fig. S3). Cell density was determined using a disposable haemocytometer (WAKEN COUNTER, Waken B Tech Co., Ltd.).

Synthesis of $CoPt@RBCG_{Co1P10pep}$

A buffer solution of $CoCl_2 \cdot 6H_2O$ and K_2PtCl_4 was added to a **$RBCG_{Co1P10pep}$**

suspension in 5P7.5 (protein concentration of 192 μg/mL, various combinations of $CoCl_2$•$6H_2O$ and K_2PtCl_4 concentrations (0.45/0.15, 0.30/0.30, 0.15/0.45, 0.30/0.10, 0.20/0.20, 0.10/0.30 mM)). The reaction mixture was stirred at 17 °C for 90 min. A buffer solution of $NaBH_4$ was added to the resulting mixture (10 equiv. relative to the total metal concentration). After stirring for 90 min at 17 °C, the mixture was centrifuged to obtain **CoPt@RBCG$_{Co1P10pep}$**. **CoPt@RBCG$_{Co1P10pep}$** was washed five times with the 5P7.5 buffer. The concentrations of Co and Pt in **CoPt@RBCG$_{Co1P10pep}$** suspensions in 5P7.5 buffer were determined using ICP-OES.

Synthesis of CoPt@RBCG

Synthesis of **CoPt@RBCG** was performed according to the procedure used for the synthesis of **CoPt@RBCG$_{Co1P10pep}$**, except that RBCG was used instead of **RBCG$_{Co1P10pep}$**.

Synthesis of M$_{0.60}$@RBCG$_{Co1P10pep}$ and M$_{0.60}$@RBCG (M = Co or Pt)

Synthesis of **M$_{0.60}$@RBCG$_{Co1P10pep}$** and **M$_{0.60}$@RBCG** (**M** = **Co** or **Pt**) was performed according to the procedure used for the synthesis of **CoPt@RBCG$_{Co1P10pep}$** and **CoPt@RBCG**. The concentration of $CoCl_2$•$6H_2O$ or K_2PtCl_4 was set to 0.60 mM.

Synthesis of CoPt NPs in the presence of Co1P10 without RBCG

Synthesis of CoPt NPs in the presence of **Co1P10** without RBCG was performed according to the procedure used for the synthesis of **CoPt@RBCG$_{Co1P10pep}$**. A buffer solution of $CoCl_2$•$6H_2O$ and K_2PtCl_4 was added to a **Co1P10** solution in 5P7.5 (**Co1P10** concentration: 2.6 μM or 260 μM; $CoCl_2$•$6H_2O$ and K_2PtCl_4 concentrations: 0.45 mM and 0.15 mM). The reaction mixture was stirred at 17 °C for 90 min. A buffer solution of $NaBH_4$ was added to the resulting mixture (10 equiv. relative to the total metal concentration). The mixture was stirred

at 17 °C for 90 min. The resulting CoPt NPs could not be harvested from the mixture by centrifugation, so the mixture was used for TEM measurements without further purification.

SQUID measurement

Freeze-dried samples of $\mathbf{Co_{0.45}Pt_{0.15}@RBCG_{Co1P10pep}}$, $\mathbf{Co_{0.45}Pt_{0.15}@RBCG}$ and RBCG were used for SQUID measurements. The samples were then packed into gelatin capsules. The field dependence of magnetisation curves were measured in the field range of -70000 Oe to 70000 Oe at 5 K. The observed magnetic moment data were corrected using diamagnetic correction.

References

1 G. Schwoch and H. Passow, *Mol. Cell. Biochem.*, 1973, **2**, 197–218.

2 H. Matsumoto, K. Okuichi, H. Imamura, K. Yasuhara, M. Kato and T. Koshiyama, *Chem. Commun.* 2022, **58**, 12220–12223.

3 H. Hiroe, M. Kawamoto, H. Imamura, T. Koshiyama, *Chem. Eur. J.* 2024, **30**, e202303749.