Supporting Information

Monolithic peptide-nucleic acid hybrid functioning as an artificial microperoxidase

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Contents

1. Synthetic procedure and analysis data

1.1 Synthesis of undecapeptide

Rink Amide AM resin (200-400 mesh, 0.62 mmol g^{-1} loading) was purchased from Merck Millipore (Darmstadt, Germany). Fmoc-protected amino acids (Fmoc-AA-OH) were obtained from Watanabe Chemical Industries, Ltd (Hiroshima, Japan). Similarly piperidine, o-(1H-benzotriazol-1-yl)-N,N,N',N'- tetramethyluronium hexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBt), and diisopropylethylamine (DIPEA) were supplied from the company. HPLC grade dimethyl formamide (DMF), dichloromethane (DCM), trifluoroacetic acid (TFA), acetic anhydride (Ac_2O), triisopropylsilane (TIS), and 1,2-ethanedithiol (EDT) were bought from Wako Pure Chemical Industries Ltd (Osaka, Japan). Other chemicals were reagent grade and used as received.

The undecapeptide were prepared by solid phase peptide synthesis (SPPS) at a 1-mmol scale in a manually operated microwave synthesis system (Discover® SP, CEM Co, Matthews, NC, USA). The corresponding amount of Rink Amide AM resin (161 mg) was settled in a reaction vessel and was swollen in 7 mL DMF for overnight prior to use. For fmoc deprotection, 0.1 M HOBt in 20%-piperidine/DMF solution was used. The activator base solution was 5%-DIPEA in DMF. The cleavage cocktail was prepared by mixing TFA (4.7 mL), TIS (0.05 mL), EDT (0.125 mL), and deionized water (0.125 mL). The synthetic procedure was as given below.

1. Wash the resin with 7 mL DMF and drain the solvent by filtration under reduced pressure. This treatment was repeated three times.
2. Fmoc deprotection of solid support. Add the fmoc-deprotection solution (7 ml). Apply microwave (MW) at 20 W The reaction temperature was set 75 °C and the reaction mixture was left to react for 3 min and then, drain the solution. Wash the resin with 7 mL DMF, swirl for 10 s, and drain the solvent. Repeat four times.
3. AA coupling with resin. Each 0.5-mmol amount of Fmoc-Glu(OtBu)-OH and HBTU were dissolved into a 4-mL portion of the activator base solution and the solution was added to the resin. Apply MW (20 W, 75 °C, 5 min) and then, drain the solvent. Wash the resin with 7 mL DMF, swirl for 10 s, and drain the solvent.

Repeat four times. As occasion arose, Kaizer test confirmed that the reaction satisfactory proceed.

4. Peptide chain elongation. Each 0.5-mmol amount of Fmoc-AA-OH and HBTU were dissolved into a 4-mL portion of the activator base solution. Repeat step 2 and 3 for each successive amino acid in the sequence in the following order: Fmoc-Val-OH, Fmoc-Thr(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ala-OH, Fmoc-Cys(Trt)-OH, Fmoc-Lys(boc)-OH, Fmoc-Gln(Trt)-OH, and Fmoc-Val-OH. Kaizer test confirmed the final coupling reaction. Wash the resin with 7 mL DMF, swirl for 10 s, and drain the solvent. Repeat four times.
5. N-terminal acetyl capping. Add the fmoc-deprotection solution (7 ml). Apply microwave (MW, 20 W, 75 °C, 3 min) and drain the solvent. Wash the resin with 7 mL DMF, swirl for 10 s, and drain the solvent. This procedure was repeated four times. Subsequently, wash the resin with 7 mL DCM, swirl for 10 s, and drain the solvent. This procedure was repeated four times. Add an 8-mL portion of 25% Ac_2O in DMF, stand for 5 min, and drain the solvent. Wash with 7 mL DCM, swirl for 10 s, and drain the solvent. Repeat four times.
6. Peptide release and side chain deprotection. Add the cleavage cocktail, swirl for 10 s, and apply MW (20 W, 38°C, 30 min). Transfer the solution cleaved peptide by filtration under reduced pressure. Prepare 34-pieces of clean 15-mL centrifuge tubes and into each tube placed was a 10-mL portion of cold diethyl ether. The equally-divided portions of the peptide solution were poured into the tubes and allow the peptide to precipitate for at least 30 min. Centrifuge the precipitated peptide for four-successive times (120,000 rpm, 30 min) at 0 °C to isolate the crude product and then, carefully decantate the supernatant. Repeat four times. The crude peptide thus obtained was dried under reduced pressure overnight.
7. Precipitation separation. Dissolve the peptide in 15 mL deionized water. Freeze the sample with liquid nitrogen and lyophilized overnight. The crude product was obtained as white precipitate in glacial ether: Yield 0.1143 g (92%).
8. Peptide analysis. The undecapeptide was examined by HPLC and was chromatographically purified on a C18-column (4.6 x 250 mm) if necessary (buffer A: 0.05% TFA in water; buffer B: 0.01% TFA in acetonitrile). HPLC

elution (1 mL min^{-1}) was initialized at 85% buffer A that was linearly changed at time to 30% (0–30 min), 20% (30–35 min), 95% (35–45 min), and 95% (45–50 min). MALDI TOFF-MS using α-cyano-4-hydroxycinnamic acid as matrix analysed the obtained products; m/z 1288.288 ($M\text{-}H^+$. $C_{52}H_{86}N_{16}O_{18}S_2$ requires 1287.47).

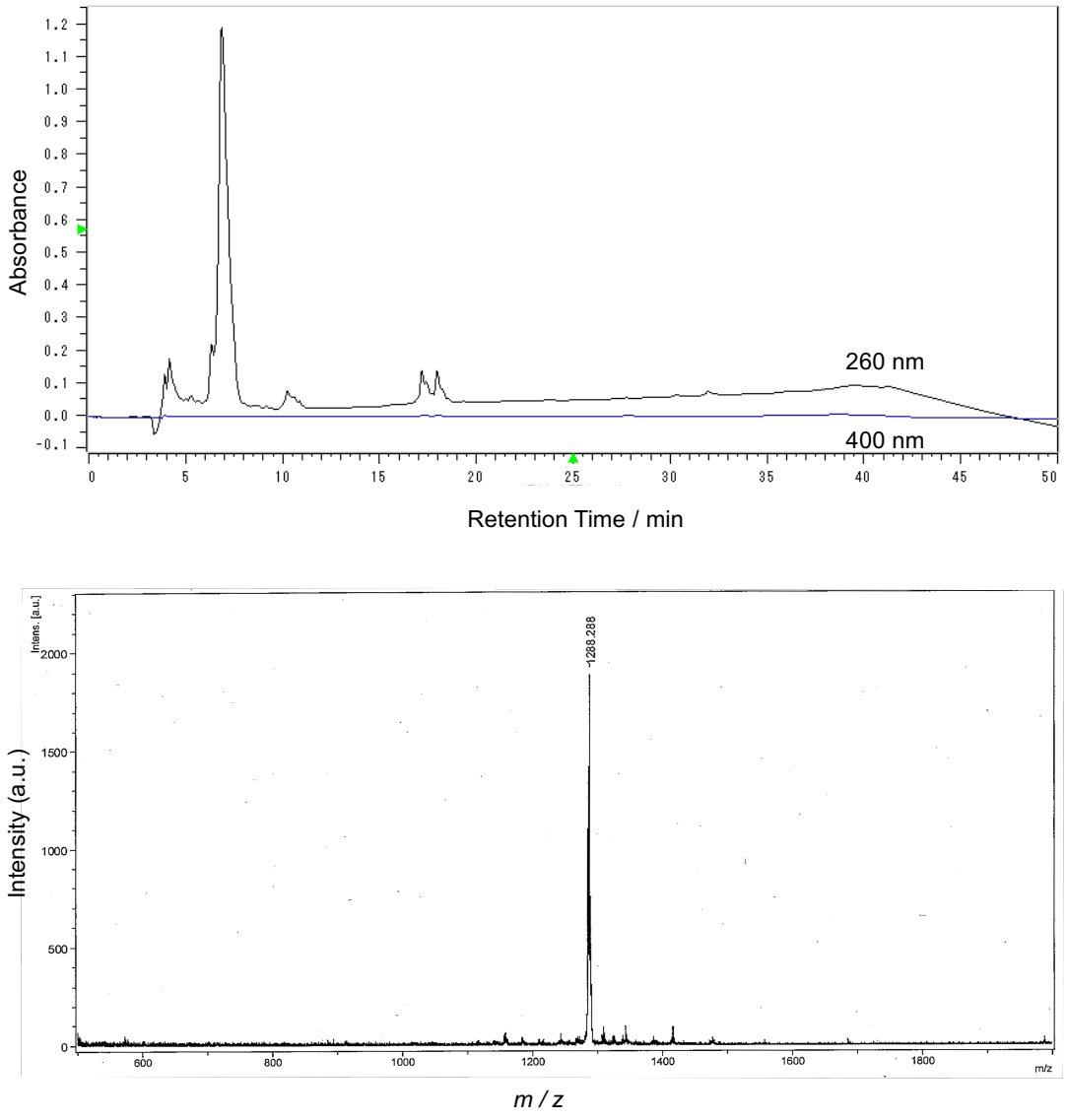

Figure S1. Results of HPLC separation (top) and MALDI-TOF-MS analysis (bottom) for undecapeptide.

1.2 Synthesis of PNA(T6)-undecapeptide conjugate

H-Rink Amide ChemMatrix® resin (35-100 mesh, typical loading ranges 0.4 and 0.7 mmol/g) and 2,6-lutidine were purchased from Sigma-Aldrich Co. LLC, St. Louis, MO, USA). Fmoc protected thymine (Fmoc-PNA-T-OH) and the spacer segment, [2-(2-(Fmoc-amino)ethoxy)ethoxy]acetic acid (F-moc-AEEA), were obtained from Link Technologies Ltd (Lanarkshire, Scotland, UK). N-Methylpyrrolidone (NMP), and 1H-benzotriazol-1-yloxy-tri(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) were bought from Watanabe Chemical Industries, Ltd (Hiroshima, Japan). Other SPPS reagents are similar as descried above. Fmoc-AA-OH monomers (0.5 M, 1 ml/coupling) and Fmoc-PNA-T-OH (0.2 M, 1 ml/coupling) were dissolved in DMF or NMP, respectively.

The PNA(T6)-undecapeptide conjugate were prepared at a 50-μmol scale. The corresponding amount of H-Rink Amide ChemMatrix® resin (167 mg) was settled in a reaction vessel and was swollen in 7 mL DMF for 45 min prior to use. An NMP solution containing 0.2 M DIPEA and 0.2 M 2,6-lutidine was prepared and used for both PNA-to-AA and PNA-to-PNA coupling reactions (1mL / coupling). Fmoc deprotection was carried out with 20% piperidine in DMF. PyBOP was dissolved in DMF to give a 0.2 M solution (1 mL / coupling). An acetic-acid (5%) / 2,6-lutidine (6%) mixed DMF solution was used for capping reaction (3 mL / PNA coupling). 0.4 M DIPEA solution in NMP was used for AA-to-AA coupling, (1 mL / coupling).

1. Wash the resin with 4 mL DMF and drain the solvent by filtration under reduced pressure. This treatment was repeated for two times.
2. Fmoc deprotection of solid support. Add the fmoc-deprotection solution (3 mL). Apply MW at 35 W. The reaction temperature was set 38 °C and the reaction mixture was left to react for 30 s and then, drain the solution. Add the deprotection solution (3 mL) and the resin was further subjected for deprotection by applying MW (47 W, 73 °C, 3 min). After draining the solution, wash with 4 mL DMF, swirl for 10 s, and drain the solvent (five successive times).
3. AA coupling with resin. Wash with 4 mL DMF, swirl for 10 s, and drain the solvent (two successive times). Add each 1 mL portion of Fmoc-Gly(OtBu)-OH, PyBOP, and DIPEA/NMP. Apply MW (17 W, 73 °C, 10 min) and then, drain the

solvent. Wash with 4 mL DMF, swirl for 10 s, and drain the solvent (five successive times).

4. PNA to AA coupling. Wash with 4 mL DMF, swirl for 10 s, and drain the solvent (two successive times). Add 3 mL deprotection solution, apply MW (35 W, 38 °C, 30 s) and then, drain. Add 3 mL deprotection solution, apply MW (35 W, 20 °C, 12 min) and then, drain. Wash with 4 mL DMF and drain (seven successive times). Add each 1 mL portion of Fmoc-PNA-T-OH, PyBOP, and DIPEA/lutidine, apply MW (17 W, 73 °C, 30 min) and then, drain. Wash with 4 mL DMF and drain (two successive times). Add 3 mL capping solution, left for react at 20 °C for 5 min and then, drain. Wash with 4 mL DMF and drain (five successive times).
5. PNA chain elongation. Wash with 4 mL DMF, swirl for 10 s, and drain the solvent (two successive times). Add 3 mL deprotection solution, left for react at 20 °C for 1 min and then, drain. Add 3 mL deprotection solution, left for react at 20 °C for 3 min and then, drain. Wash with 4 mL DMF, swirl for 10 s, and drain the solvent (seven successive times). Add each 1 mL portion of Fmoc-PNA-T-OH, PyBOP, and DIPEA/lutidine, apply MW (17 W, 73 °C, 30 min) and then, drain. Wash with 4 mL DMF, swirl for 10 s, and drain (two successive times). Add 3 mL capping solution, left for react at 20 °C for 5 min and then, drain. Wash with 4 mL DMF and drain (five successive times). Repeat this step five successive times using Fmoc-PNA-T-OH. Finally, the resin was subjected for Fmoc-AEEA coupling to give the PNA sequence intended.
9. AA to AEEA coupling. Wash with 4 mL DMF, swirl for 10 s, and drain the solvent (two successive times). Add 3 mL deprotection solution, left for react at 20 °C for 1 min and then, drain. Add 3 mL deprotection solution, left for react at 20 °C for 3 min and then, drain. Wash with 4 mL DMF, swirl for 10 s, and drain the solvent (seven successive times). Add each 1 mL portion of Fmoc-Glu(OtBu)-T-OH, PyBOP, and DIPEA/lutidine, apply MW (17 W, 73 °C, 10 min) and then, drain. Wash with 4 mL DMF, swirl for 10 s, and drain (five successive times).
10. Peptide chain elongation. Each Fmoc-AA-OH was dissolved in DMF to give a 0.125 M solution. Repeat step 9 for each successive AA in the sequence in the

following order: Fmoc-Val-OH, Fmoc-Thr(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ala-OH, Fmoc-Cys(Trt)-OH, Fmoc-Lys(boc)-OH, Fmoc-Gln(Trt)-OH, and Fmoc-Val-OH.

11. N-terminal deprotection. Wash with 4 mL DMF, swirl for 10 s, and drain the solvent (two successive times). Add 3 mL deprotection solution, left for react at 20 °C for 3 min and then, drain. Add 3 mL deprotection solution, left for react at 20 °C for 12 min and then, drain. Wash with 4 mL DMF, swirl for 10 s, and drain the solvent (seven successive times).
12. Peptide release and side chain deprotection. Add the cleavage cocktail, swirl for 10 s, and left to react for 4 h at room temperature. Transfer the solution by filtration under reduced pressure. The resin was washed with 2 mL TFA and the solvent was transferred to combine with the first-cleaved fraction. Prepare 4-pieces of clean 15-mL centrifuge tubes and into each tube placed was a 10-mL portion of cold diethyl ether. The equally-divided portions of the peptide solution were poured into the tubes and allow the peptide to precipitate for at least 30 min. Repeat the centrifuge the precipitated peptide for four-successive times (120,000 rpm, 30 min) at 0 °C to isolate the crude product and then, carefully decantate the supernatant. Repeat the whole procedure one more time to obtain the whole recoverable quantity of the product. The crude peptide thus obtained was dried under reduced pressure overnight.
13. Precipitation separation. Dissolve the peptide in 15 mL deionized water. Freeze the sample with liquid nitrogen and lyophilized overnight. The crude product was obtained as white precipitate in glacial ether: Yield 0.213 g (140%).
14. Peptide analysis. The PNA(T6)-undecapeptide conjugate was examined by HPLC and was chromatographically purified on a C18-column (10 x 250 mm) if necessary (buffer A: 0.05% TFA in water; buffer B: 0.01% TFA in acetonitrile). HPLC elution (3 mL min^{-1}) was initialized at 85% buffer A that was linearly changed at time to 30% (0–30 min), 20% (30–35 min), and 20% (35–50 min). MALDI TOFF-MS using α-cyano-4-hydroxycinnamic acid as matrix analysed the obtained products; m/z 3046.254 ($M\text{-}H^+$. $C_{124}H_{183}N_{42}O_{45}S_2$ requires 3044.28). An abasic compound produced by elimination of two thymine residues upon ionization ($M\text{-}H^+$. $C_{114}H_{175}N_{38}O_{41}S_2$ requires 2796.22) that further decomposed

in part, may gave a peak at m/z 2779.438.

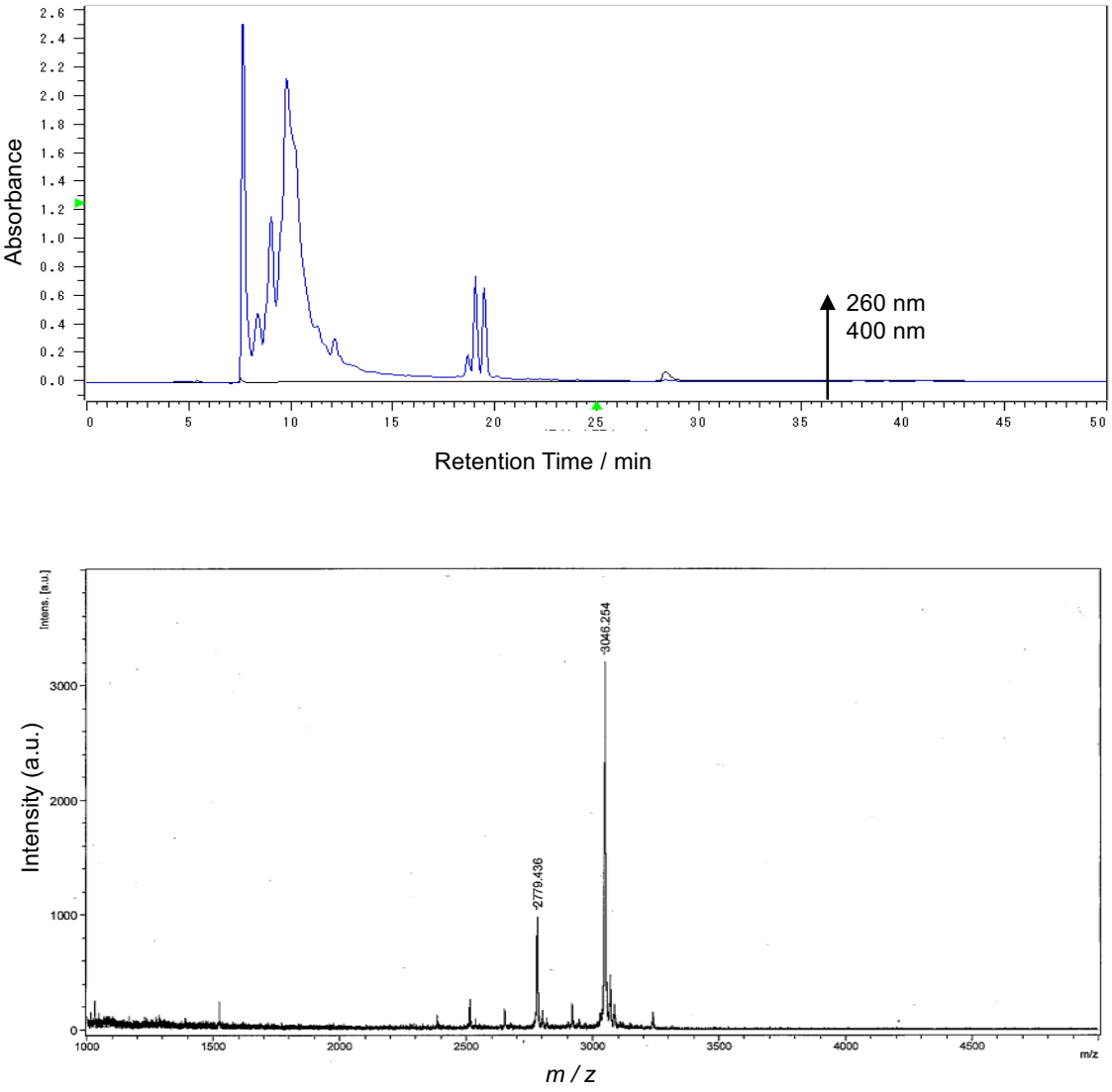

Figure S2. Results of HPLC separation (top) and MALDI-TOF-MS analysis (bottom) for PNA(T6)-undecapeptide conjugate.

*1.3 Reconstitution of **NAcMP***

For the protein-reconstitution experiments, hemin and 2-hydroxy-1-[4-(2-hydroethoxy)phenyl]-2-methylpropan-1-one (photoinitiator) were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Dithiothreitol (DTT) were supplied from Wako Pure Chemical Industries Ltd (Osaka, Japan). For control measurements, naturally-derived microperoxydase-11 was bought from Sigma-Aldrich Japan Co. LLC (Tokyo, Japan). Other chemicals were reagent grade and used as received.

The undecapaptide was reconstituted with the prosthetic group by using thiol-en click reaction[1–4]. A weighed amount of the undecapeptide (19.4 mg, 15.1 μmol) was dissolved into 3.5-mL portion of aqueous 50%-THF solution that was, by slowly bubbling nitrogen gas, deoxygenated beforehand. The solution was combined with a 200-μL volume of aqueous 0.1 M NaOH solution containing 3.2-mg of hemin (4.9 μmol) and into the reaction mixture, was added each one-third microspatula full of the photoinitiator, DTT, and sodium dithionite. The reaction was allowed to proceed by exposing to UV light (365 nm, 40 mW cm^{-2}) for 60 min at RT. The undecapeptide in the corresponding holo structure that was identified by comparison experiments using naturally-derived microperoxidase-11, was chromatographically purified on a C18-column. The HPLC conditions were the same as those used for undecapaptide. The fractions that were eluted from 12.5 min to 17.5 min, were combined and were subjected for centrifugal concentration. This resulted trace amount of red-brown powder after lyophilisation: Yield trace. We obtained, as a typical case, 1.1%-of-yield when 5-μmol of apo-**NAcMP** was subjected for the reaction and the solution was purified by HPLC in 4 portions. MALDI TOFF-MS using α-cyano-4-hydroxycinnamic acid as matrix analysed the obtained products; *m/z* 1903.41 (M^+. $C_{86}H_{118}FeN_{20}O_{22}S_2$ requires 1902.75) and additionally a fragmentation peak at *m/z* 1287.812 (M-H^+ for **NAcMP**, $C_{52}H_{87}N_{16}O_{18}S_2$ requires 1287.58), which further aggregates with the primary component to give a peak at *m/z* 3191.746 (M^+ for $C_{138}H_{204}FeN_{36}O_{40}S_4$ requires 3189.33).

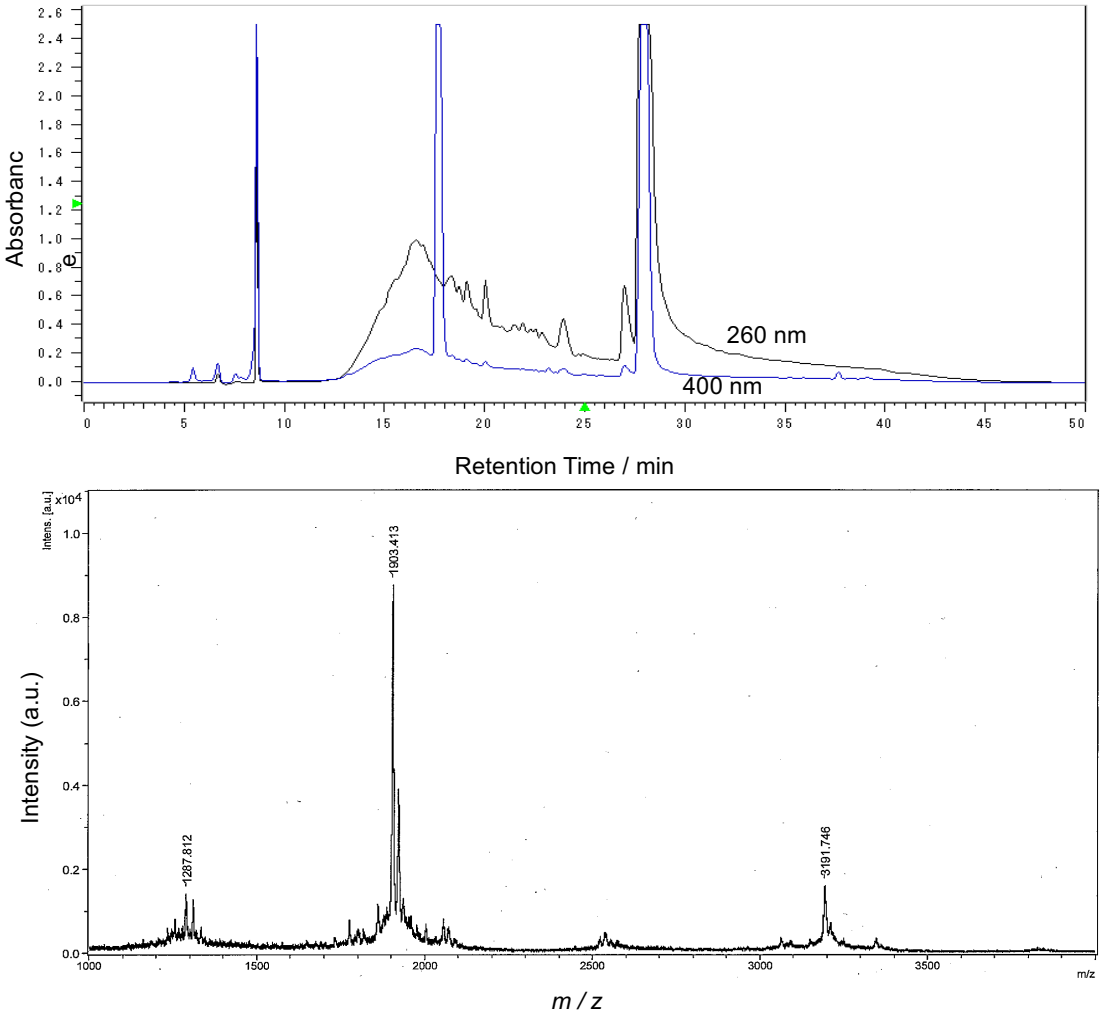

Figure S3. Results of HPLC separation (top) and MALDI-TOF-MS analysis (bottom) for **NAcMP**.

1.4 Reconstitution of ***MP-PNA(T6)***

The PNA(T6)-undecapeptide conjugate was reconstituted with the prosthetic group in a similar way that used for MP. A 38.4 mg (12.6 μmol) portion of the PNA(T6)-undecapeptide conjugate was dissolved into 3.5-mL portion of aqueous 50%-THF solution that was, by slowly bubbling nitrogen gas, deoxygenated beforehand. The solution was combined with a 200-μL volume of aqueous 0.1 M NaOH solution containing 2.6-mg of hemin (4.0 μmol) and into the reaction mixture, was added each one-third microspatula full of the photoinitiator, DTT, and sodium dithionite. The reaction was allowed to proceed by exposing to UV light (365 nm, 40 mW cm^{-2}) for 60 min at RT. The PNA(T6)-undecapeptide conjugate in the corresponding holo structure was chromatographically purified on a C18-column. The HPLC conditions were the same as those used for PNA(T6)-undecapeptide conjugate. The fractions that were eluted from 15 min to 17.5 min, were combined and were subjected for centrifugal concentration. This resulted trace amount of red-brown powder after lyophilisation: Yield trace. MALDI TOFF-MS using α-cyano-4-hydroxycinnamic acid as matrix analysed the obtained products; *m/z* 3662.60 ($M\text{-}H^+$. $C_{158}H_{215}FeN_{46}O_{49}S_2$ requires 3659.45). An abasic compound produced by elimination of two thymine residues upon ionization ($M\text{-}H^+$. $C_{148}H_{206}FeN_{42}O_{45}S_2$ requires 3411.39) that further decomposed in part, may give a peak at *m/z* 3395.652. The fragmentation peak at *m/z* 3048.039 is ascribed to the apo-peptide.

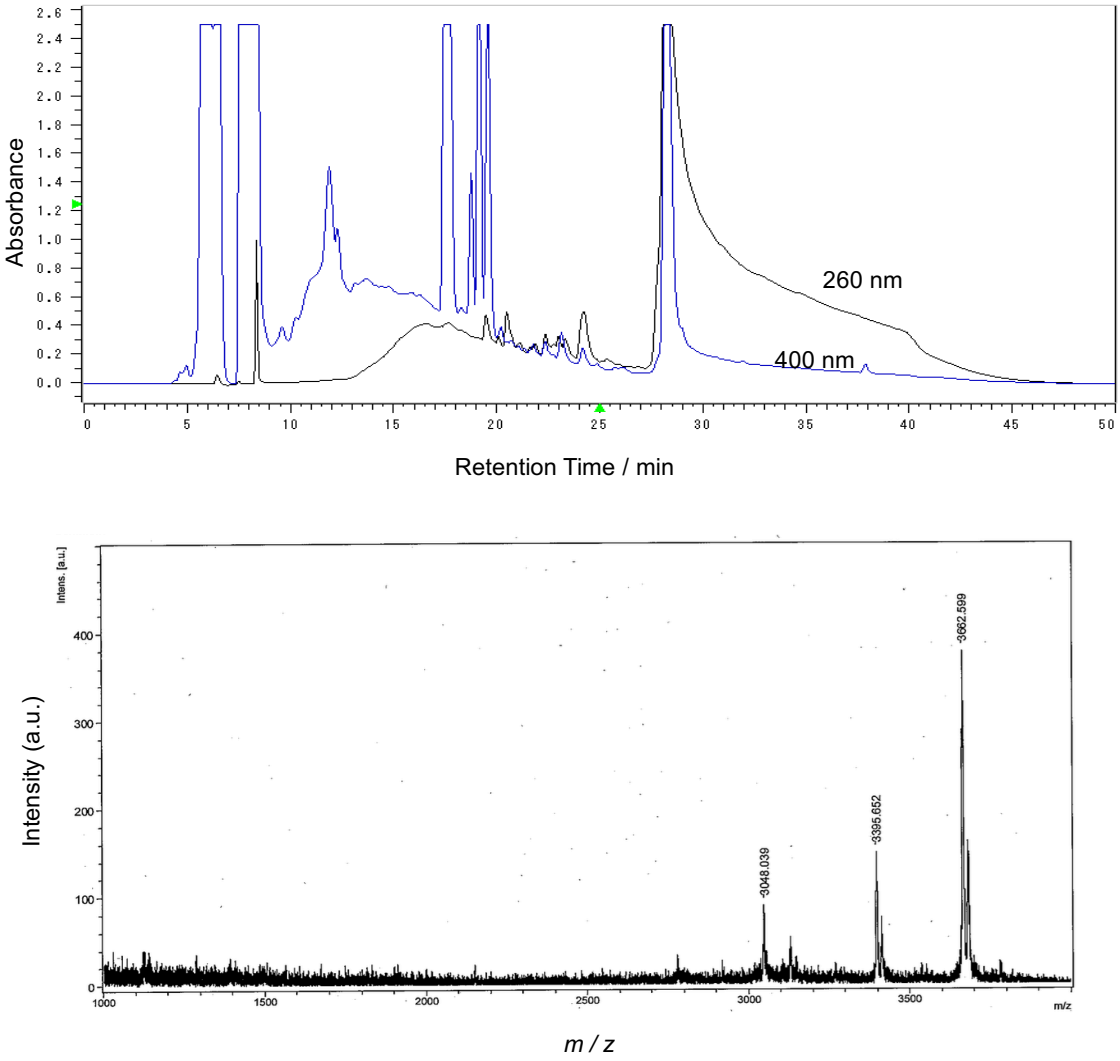

Figure S4. Results of HPLC separation (top) and MALDI-TOF-MS analysis (bottom) for **MP-PNA(T6)**.

2. *UV-vis spectra for NAcMP and MP-PNA(T6) in ferric- and ferrous-haem states*

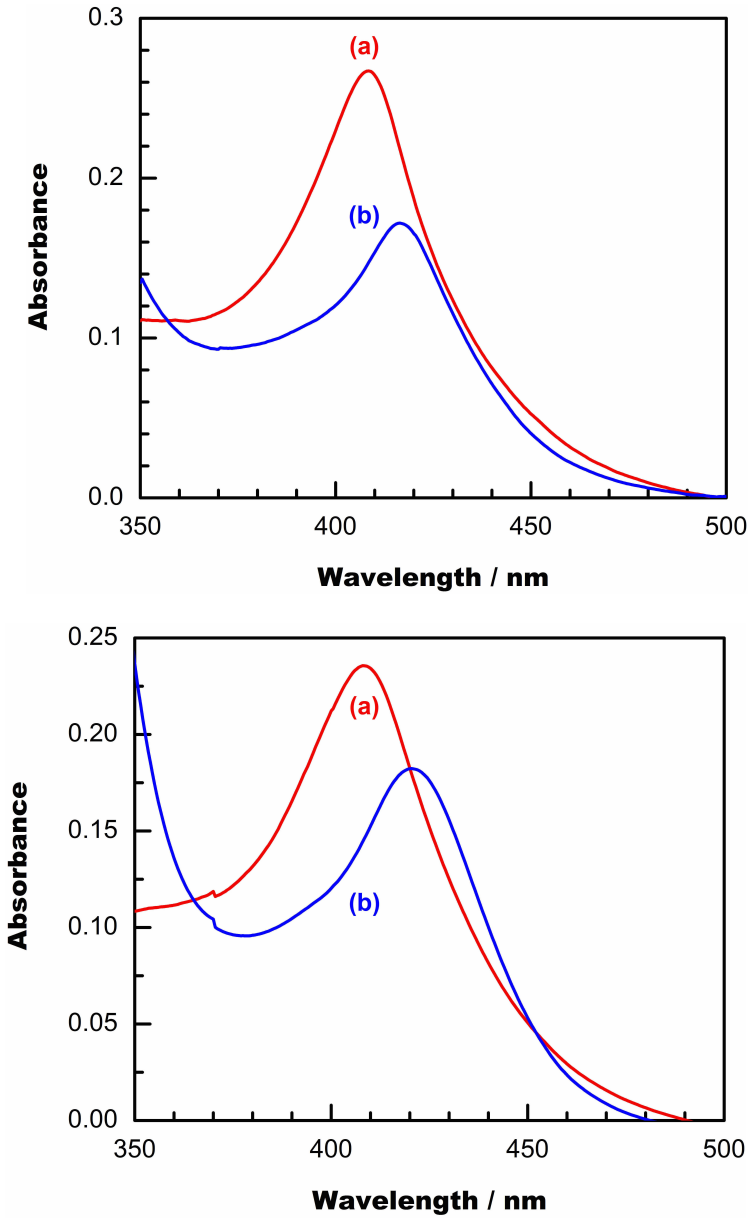

Figure S5. UV-vis spectra for **NAcMP** (top) and **MP-PNA(T6)** (bottom) either in ferric- (**a**) or ferrous-heme (**b**) state. Each sample contains $2x10^{-5}$ M either of **NAcMP** or **MP-PNA(T6)** dissolved in 0.01 M phosphate buffer (pH 7). For reduction of the ferric-heme species, a microspatula full of dithionite was added into the cell directly. The path-length of the cell was 0.2 cm and temperature was 25 °C.

3. CD spectra for natural MP11, NAcMP, and MP-PNA(T6)

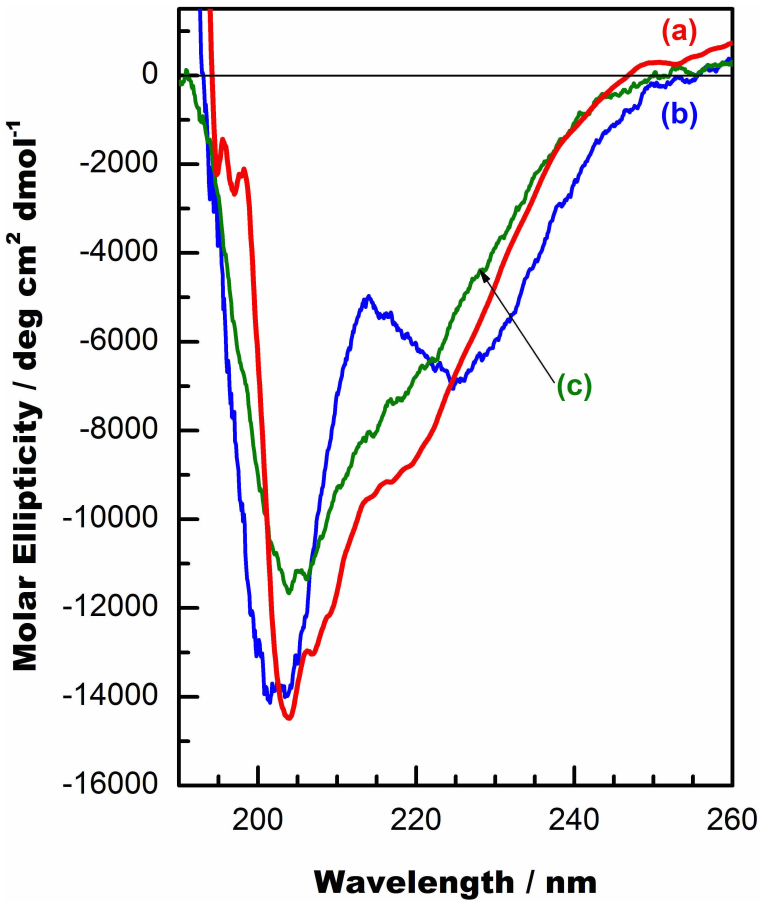

Figure S6. CD spectra for 87 µM **MP-PNA(T6)** (**a**), 64 µM **NAcMP** (**b**), and 67 µM native MP11 (**c**) dissolved in TFE. The path-length of the cell was 1 cm and temperature was 25 °C.

4. Peroxidase-activity measurements

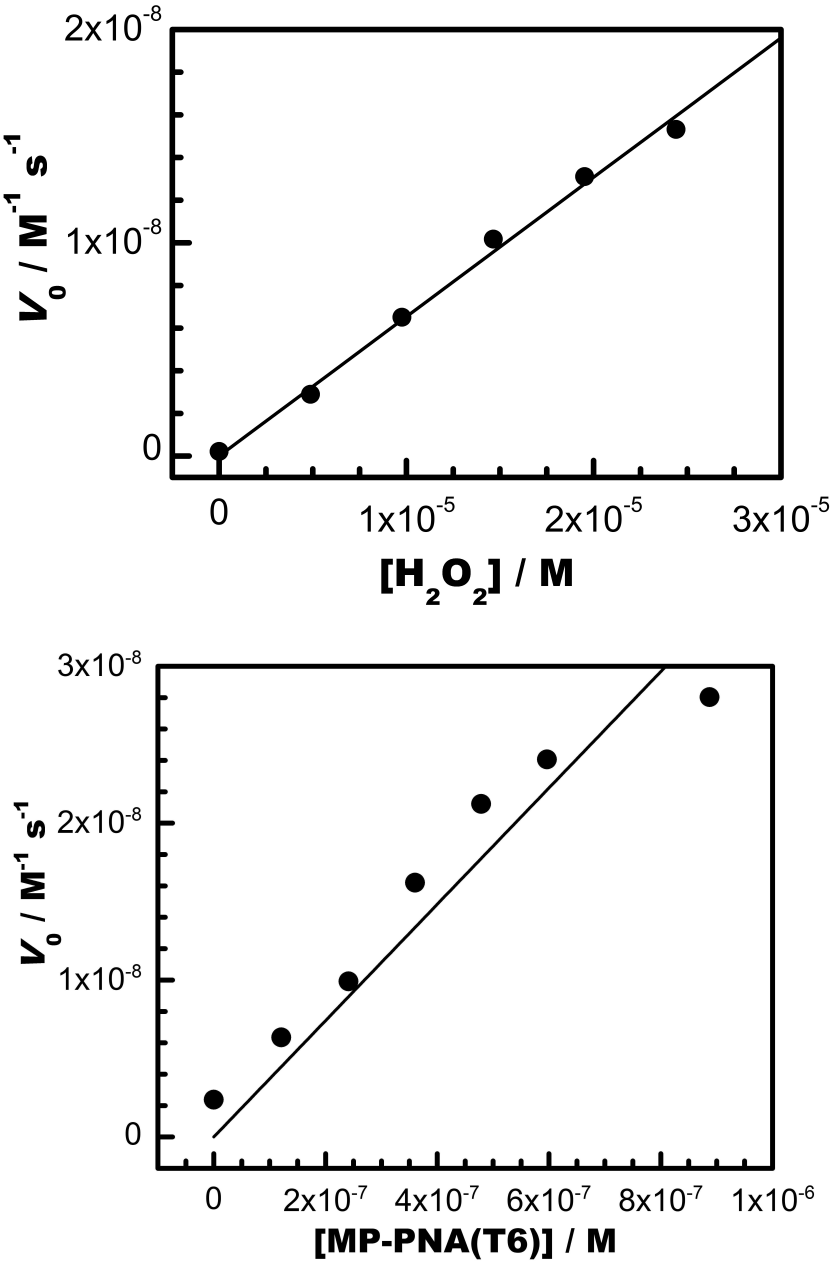

Figure S7. Relationship between TMBZ oxidation activity and concentration either of H_2O_2 (top) or **MP-PNA(T6)** (bottom). The assay solution contains 0.14 mM TMBZ (0.01 M phosphate buffer, pH 7) and either of 40 μM **MP-PNA(T6)** (top) or 15 μM H_2O_2 (bottom) and temperature was 37 °C.

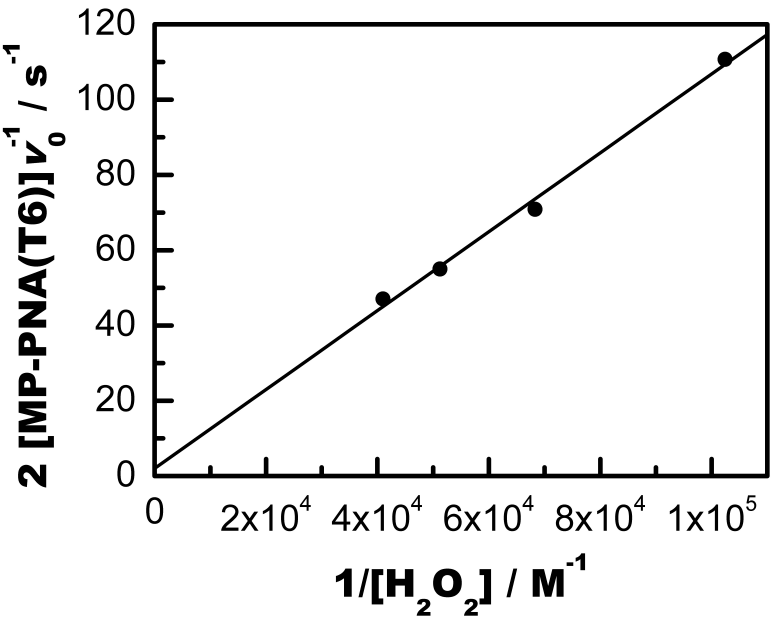

Figure S8. Plots according to the steady-state kinetics, Eq. (4), for **MP-PNA(T6)** obtained with various H_2O_2 concentrations. The assay solution (0.1 M phosphate buffer, pH 7) consists of 0.39 μM **MP-PNA(T6)**, 0.14 mM TMBZ, and 15 μM H_2O_2.

5. *CVs for* $Fe(CN)_6^{4-}$ on a bare Au electrode and the e16s-Au electrode

A model ALS 750 potentiostat (BAS Inc., Tokyo, Japan) was used for measurements. Gold disk electrodes (ϕ1.6 mm, BAS Inc.) were used in combination with a Pt-wire counter electrode and an Ag/AgCl electrode (3 M NaCl, BAS Inc.). Atop a cleaned Au electrode was placed a 10-μL portion of aqueous **e16s** solution (50 μM in 100 mM KCl). After evaporation of the solvent at room temperature, the electrode was washed thoroughly with 100 mM KCl solution and was used as a sensing interface. Attachment of e16s was confirmed by comparing the CVs for $[Fe(CN)_6]^{4-}$ (10 mM in 100 mM KCl) obtained by measuring at room temperature before and after the treatment (**Figure S9**).

For the sandwich hybridization experiments, a 100-μM solution of **MP-PNA(T6)** containing 5 mM tris–HCl (pH 8.0) and 100 mM KCl was prepared. The DNA sample solutions including **t13**, **m13**, and **sc13** were similarly prepared. A 50-μL portion of **MP-PNA(T6)** solution was mixed with either of a 50-μL portion of sample DNA solution and the solution was kept at 90 °C for 10 min. Then, the solutions were allowed to cool gradually for annealing and stored at 3 °C as stock sample solutions. Then, the sensing electrode was immersed in a 50-μL of stock sample solution at 3 °C for 24 h. Then the interface was rinsed with 100 mM KCl solution at 3 °C for washing away the non-specific binding. Target DNA responses were collected by measuring CVs of the sensing interface in 0.1 M phosphate buffer solution (pH 7.0) at 3 °C. For measurements of the H_2O_2-related electrocatalytic reaction, CVs of the sensing interface were obtained in the presence of 0.33 μM of H_2O_2 in the buffer solution. The room temperature was kept at 22 ± 2 °C during the measurements.

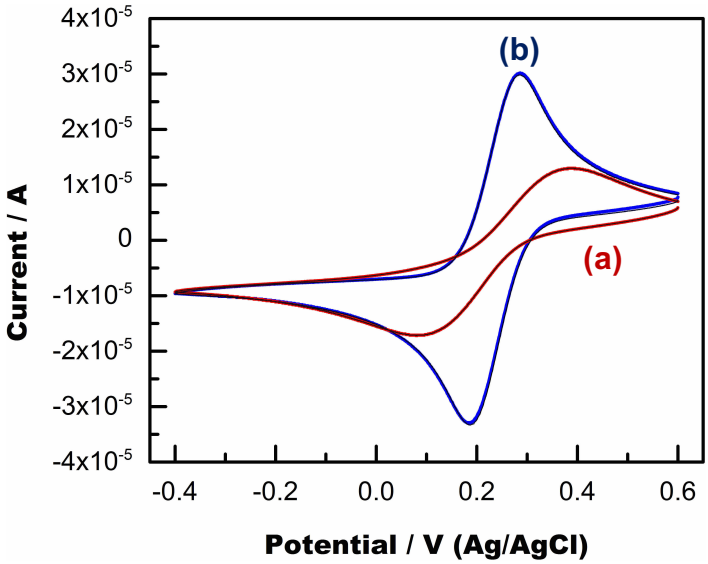

Figure S9. CVs for 10 mM $Fe(CN)_6^{4-}$ (0.1 M KCl) on a bare Au (**a**) and the e16s-Au electrode (**b**). Measurement conditions: scan rate 100 mV s^{-1}, temperature 3 °C.

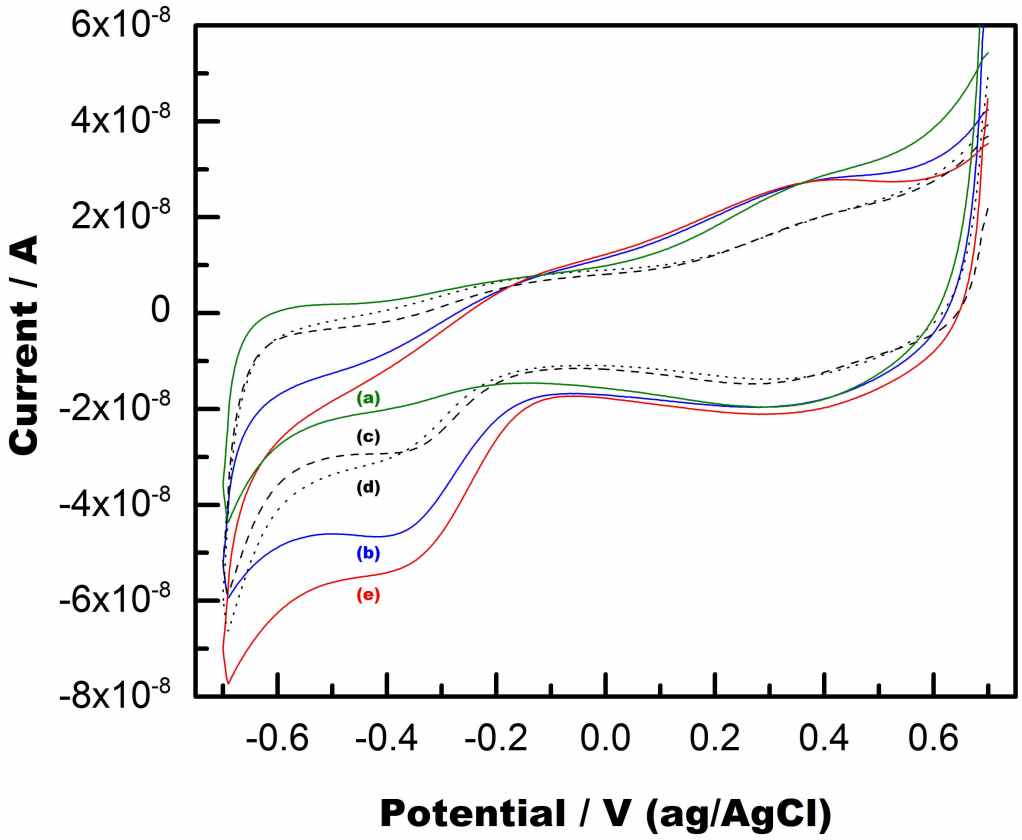

Figure S10. Full pictures of CVs shown in Fig. 3. Keys are the same as those in Fig. 3.

6. References

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