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# Dual functional peptide carrying *in vitro* selected catalytic and binding activities†

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When minimal functional sequences are used, it is possible to integrate multiple functions on a single peptide chain, like a "single stroke drawing". Here a dual functional peptide was designed by combining *in vitro* selected catalytic and binding activities. For catalytic activity, we performed *in vitro* selection for a peptide aptamer binding to hemin by using ribosome display and isolated a peptide that had peroxidase activity in the presence of hemin. By combining the selected catalytic peptide with a peptide antigen, which can be recognized by an antibody, an enzyme-antibody conjugate-like peptide was obtained. This study demonstrates a successful strategy to create dual functionalized peptide chains for use in immunoassays.

## Introduction

Joining different biological molecular functions by chemical or biological means is important for the development of new tools for biotechnology and medicine. Many bioconjugates such as antibody-enzyme and antibody-drug conjugates have already been devised and used. Conversely, the minimal components (peptides) carrying the functions of antibodies and enzymes (proteins) have also been investigated as replacements for these biological molecules to simplify design and overcome problems such as difficulties in mass production and thermal instability.[1-3] When minimal sequences are used, it is possible to integrate multiple functions on a single peptide chain like "single stroke drawing".

Enzyme immunoassays are powerful analytical tools for the quantitative detection of target molecules in complex media.[4] For these analyses antibody and enzyme are conjugated to provide molecular recognition and signal amplification, respectively. Although some bi-functional peptides incorporating recognition and signal amplification functions have been reported for analytical applications,[5, 6] some additional operations such as cleavage of peptide or release of probe processes were required. Therefore, in this study a more convenient catalytic component was designed to make use of general methods comparable with the usual enzyme immunoassay process. Peroxidases are widely used as biocatalysts for bioreactors and biosensors[7] or for conjugation with antibodies.[8, 9] Peroxidases are heme enzymes and utilize hemin as a cofactor to catalyze the oxidation of a variety of substrates.[10, 11] Several groups have succeeded in preparing catalytic antibodies and oligonucleotides with peroxidase activity,[12-19] and we previously reported *in vitro* selected DNA and RNA aptamers that bind hemin and exhibit peroxidase activity.[20-22] However, peptide aptamers showing peroxidase activity have not been previously reported. Therefore, we performed *in vitro* selection of peptide aptamers for hemin for the first time using ribosome display. We used ribosome display because the diversity of a random peptide library is higher than that of phage display and thus we were able to isolate a high affinity peptide using this method.[23] The selected peptide aptamers were characterized and their peroxidase activity was investigated. To develop a dual functionalized peptide chain, the selected aptamer sequence with the best binding affinity to hemin was conjugated with a model molecular recognition sequence (FLAG sequence), and after forming a complex with hemin, it was used to detect an anti-FLAG antibody as a model analyte on a membrane, as illustrated in Fig. 1.

## Materials and methods

### Materials

Hemin-agarose beads were obtained from Sigma Co. (St. Louis, MO, USA). Dynabeads MyOne Streptavidin C1 and magnet were obtained from Invitrogen Co. (Carlsbad, CA, USA). Deoxyribonucleotide triphosphate (dNTP) monomers and AmpliTaq DNA polymerase with reaction buffer were purchased from Roche Diagnostics Co. (Basel, Switzerland). All ssDNA oligonucleotides used for library generation were synthesized using an automated DNA synthesizer (H-8-SE model, Gene World Co., Tokyo, Japan) by typical phosphoramidite chemistry.

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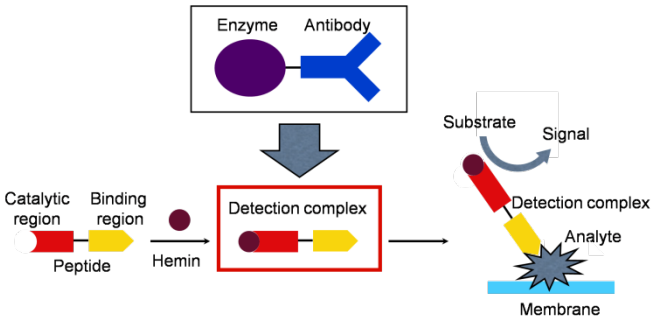


Fig. 1 A dual functionalized peptide chain functions like a peroxidase-labeled antibody. The selected aptamer sequence (catalytic region) was conjugated with the molecular recognition sequence (binding region) and formed a detection complex with hemin. The complex was used to detect the analyte on a membrane by turning over an ECL substrate to generate light.

Hemin purchased from Sigma Co. was used without further purification. 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and bovine serum albumin (BSA), anti-FLAG antibody (monoclonal ANTI-FLAG, BioM2, produced in mouse, purified immunoglobulin) were obtained from Sigma Co. (St. Louis, MO, USA). 2-morpholinoethanesulfonic acid hydrate (MES) was purchased from Chemical Dojin Co. (Kumamoto, Japan); Triton X-100 detergent was obtained from Calbiochem Co. (Darmstadt, Germany), and Tween 20 was purchased from MP Biomedicals, Inc. (IIIkrich, France). Horseradish peroxidase (HRP), hydrogen peroxidase and other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

### *In vitro* selection

We designed and used a single-stranded DNA library (Operon, Tokyo, Japan) that contained an $(NNK)_{10}$ random sequence to prepare a peptide library.

The selection method was performed as previously reported.[24] Initially, one round of polymerase chain reaction (PCR) amplification was conducted using a GXL enzyme kit (Qiagen, Tokyo, Japan). This resulted in double-stranded DNA sequences, which were digested withSfiI (New England Biolabs, Ipswich, MA, USA) and ligated into the 13Trx plasmid vector (Fig. S1). Fifteen pmoles of random sequence DNA ($10^{13}$ diversity) was ligated to the plasmid and amplified by 10 rounds of PCR. Using the PURE system (Wako Pure Chemicals, Osaka, Japan), *in vitro* transcription and translation was conducted to form the peptide-ribosome-mRNA complex.

Hemin-immobilized beads (10 µL, 3.5–6 × $10^7$) were mixed with the 50µL PURE system solution containing 60 pmoles of peptide-ribosome-mRNA complex. After incubation in 600 µL buffer (0.1% Tween 20, 60 Mm Tris, 180 mM NaCl, 60 mM magnesium acetate, pH 6.5) at 4°C for 1 h, the suspension was centrifuged and washed with water six times. The 100 µL suspension of 0.1% w/v beads/peptide-ribosome-mRNA complex was treated with 10 µL of 10 mM EDTA to release mRNA. The mixture was centrifuged and the supernatant containing mRNA was drawn out and purified using RNeasy kits. The mRNA was collected for *in vitro* reverse transcription and the resulting cDNA was amplified. These processes made up one round. After four rounds, three duplicated or partially duplicated sequences were found in the 96 sequenced clones, so no further rounds of selection were carried out.

### Peptide synthesis

As listed in Table 1, the sequences Xu1, Xu2, and Xu6 were synthesized by standard Fmoc solid-phase synthesis at the RIKEN Brain Science Institute.Xu6-FLAG, the conjugate of peroxidase sequence Xu6, and the molecular recognition sequence FLAG, were synthesized by GenScript USA Inc.

### UV/Vis measurement

The ability of the four peptides Xu1, Xu2, Xu6 and Xu6-FLAG to form a complex with hemin was investigated by UV/Vis spectroscopy. The stock solutions of 10 mM Xu1, Xu6 and Xu6-FLAG were prepared in milli-Q water while Xu2 was prepared in dimethylformamide (DMF), and 1 mM hemin was dissolved in 30 mM NaOH. All of the peptide concentrations in this study were calculated on mass. The absorption spectra of 50 µL of 2 µM hemin in the absence and presence of various concentrations of Xu1, Xu2, Xu6 and Xu6-FLAG were obtained in 40KT buffer (50 mM 2-morpholinoethanesulfonic acid hydrate (MES); 100mMTris acetate; 40 mM potassium acetate; 1% DMSO; 0.05% Triton X-100, pH 6.5) using a V-550spectrophotometer (Jasco, Tokyo, Japan) (scan speed, 200 nm per minute; response, medium; bandwidth, 1.0 nm). Each sample was measured three times and average spectra are reported.

### CD measurement

The binding of the peptides Xu1, Xu6 and Xu6-FLAG with hemin was also confirmed by circular dichroism (CD) spectroscopy. The measurements were performed using a J-720 spectrophotometer (Jasco, Tokyo, Japan). Seven hundred microliters of 10 µM Xu1, Xu6 or Xu6-FLAG in the absence and presence of 20 µM hemin in reduced PBS buffer (1.47 mM $KH_2PO_4$; 8.06 mM $Na_2HPO_4$, pH 6.6) was measured using a 5 mm cuvette (scan type, continuous; scan speed, 100 nm per minute; response time, 1.0 s; bandwidth, 1.0 nm; accumulation, 20). As the stock solution of hemin contained 30 mM NaOH, to compare the spectra of unbound peptides and hemin complexes under exactly the same conditions, the corresponding amount of additional NaOH was added to the Xu1, Xu6 and Xu6-FLAG solutions when obtaining spectra of the pure peptides. Each sample was measured three times and average spectra are reported. Xu2 was not measured because the solvent DMF increased the short wavelength limit of CD and there was no meaningful spectrum in the detectable region.

### Catalytic activity measurement

The peroxidase activity of hemin, peptide-hemin complexes and HRP was evaluated by determining the rates of ABTS oxidation. The stock solution of 50 mM ABTS was prepared in milli-Q water. The peptides (final concentration 20 µM) and hemin (final concentration 0.25 µM), or HRP (final concentration 200 pM) were placed in 40KT buffer; after addition of ABTS (final concentration 6 mM) and $H_2O_2$ (final concentration 1.8 mM) to the peptide-hemin solution (total volume 80 µL), the time dependency of the absorbance at 414 nm was monitored on a V-550 spectrophotometer (response, medium;

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bandwidth, 2.0 nm). The rate curves shown are an average of three measurements for each case.

**Molecular recognition experiment**

The molecular recognition of the selected peptide aptamer Xu6 conjugated with the molecular recognition sequence FLAG (DYKDDDDK) was studied using a dot blot.

First, the catalytic activity of Xu6-FLAG and hemin complex on the enhanced chemiluminescence (ECL) substrate was confirmed as follows. A PVDF blotting membrane (pore size: 0.2μm) was immersed in methanol followed by blotting buffer (25 m MTris, 192 mM Glycine, 20% (v/v) methanol) and placed on a wet filter paper to avoid it drying out. Two-microliter portions of 0.25 μM hemin and various concentrations of Xu6-FLAG in milli-Q water were then placed on to the membrane. After it had dried out, the membrane was immersed in methanol followed by blotting buffer. An ECL kit (ECL Prime Western Blotting Detection Regent, GE Healthcare, Buckinghamshire, UK) was then used for light development of the membrane and a chemiluminescence image of the membrane surface was taken by a Light-Capture with ATTO Cooled CCD Camera System (ATTO Corporation, Tokyo, Japan). The image was analyzed using the ImageJ64 software package, and the integrated densities of the analyte spots were obtained.

Subsequently, the molecular recognition ability of Xu6-FLAG was investigated. A PVDF membrane (Immobilon-P Transfer Membrane, pore size: 0.45 μm) treated with the same method as above was placed on wet filter paper, and 2 μL portions of 1 mg $mL^{-1}$ anti-FLAG antibody with dilution ratios of 1:1, 1:2, 1:4, 1:8 and 1:16 in PBS buffer were then placed on to the membrane, respectively. Two-microliter portions of 1 mg $mL^{-1}$ gelatin and 1 mg $mL^{-1}$ BSA with the same dilution ratios were also placed on to the membrane as negative controls. After it had dried out, the membrane was immersed in methanol followed by blotting buffer. The membrane was then immersed in blocking buffer (2.5% normal horse serum, Vector laboratories, Inc., Burlingame, CA) and shaken for 1 h at room temperature to block the occurrence of any nonspecific binding. The membrane was then briefly washed with TBS-T buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4) and placed on parafilm to keep it wet. Six hundred microliters of 100 μM Xu6-FLAG and 2 μM hemin complex was then added to the membrane to cover the whole test area. After being incubated for 2 h at room temperature, the membrane was washed twice with TBS-T buffer for 5 min each time, and an image of the surface of the membrane was taken as described above. Furthermore, to confirm the catalytic activity of the Xu6-FLAG-hemin complex using a dot blot, three PVDF membranes (Immobilon-P Transfer Membrane, pore size: 0.45 μm) treated as described above were placed on wet filter paper, and 2 μL portions of 1 mg $mL^{-1}$ anti-FLAG antibody were spotted onto each membrane. After blocking, 100 microliters of 100 μMXu6-FLAG with 2μM hemin, 100 microliters of 100 μM Xu6-FLAG alone, and 100 microliters of 2 μM hemin alone were added to the three membranes respectively. After being incubated and washed as described above, an image of the surfaces of the three membranes was taken.

## Results and discussion

Table 1 Peptide sequences prepared by standard Fmoc solid-phase synthesis.

| Peptide | Sequence |
|---|---|
| Xu1 | YRLPHVVAPG |
| Xu2 | CAVVDWFYEF |
| Xu6 | SAECTRVQCH |
| Xu6-FLAG | SAECTRVQCHGGSDYKDDDDK |

***In vitro* selection**

For the *in vitro* selection of peptide aptamers, we designed a single-stranded DNA library that contained an $(NNK)_{10}$ random sequence to prepare a peptide library. *In vitro* selection was performed on hemin-immobilized beads for four rounds after which one fully duplicated sequence (Peptides 13 and 23) and two partially duplicated sequences (Peptides 8 and 28, VVAP; Peptides 20, 33 and 51, SAE) were found in the 96 clones sequenced (see ESI†, Table S1). Based on the frequency of the clones, the four peptides Xu1, Xu2, Xu6, and Xu6 conjugated to the molecular recognition sequence FLAG were prepared by solid phase synthesis (Table 1). Mass spectra of the synthesized peptides are shown in Figures S2–5.

**Interaction of selected peptides and hemin**

The interaction of the selected peptides Xu1, Xu2, Xu6 and Xu6-FLAG with hemin was investigated both by UV/Vis and circular dichroism (CD) spectroscopy. Fig. 2a shows the UV/Vis spectra of Xu1, Xu2, Xu6 binding to hemin in 40KT buffer. A change in the Soret absorption of hemin was observed in the presence of Xu6 but not in the presence of Xu1 and Xu2. Peak absorbance changes of the UV-visible spectrum of hemin in the presence of various concentrations of peptides Xu1, Xu2 and Xu6 is shown in Fig. 2b. The association constant of Xu6 and hemin was determined to be 26.4 ± 1.8 μM. Conformational analysis of the interaction between the peptide aptamers Xu1 and Xu6 and hemin (CD spectroscopy, Fig. S6) revealed that the aptamers exhibited a negative band at 190–220 nm, indicating a random coil conformation. When hemin was added to the solution, a slight change in this band was observed for Xu6, which confirmed the existence of the interaction between this aptamer and hemin. The peptide Xu2 could not be investigated by CD because it required dissolution in DMF, which interferes with the CD signal at low wavelengths.

**Catalytic activity of selected peptides**

The peroxidase-like activity of the peptides was investigated by the addition of ABTS and $H_2O_2$ to the solutions of Xu1, Xu2 or Xu6 with hemin (Fig.2c). In this assay, peroxidase activity causes reduction of $H_2O_2$. This, in turn, induces ABTS oxidation, resulting in the formation of the ABTS radical cation, which has an absorption maximum at 414 nm. In the case of hemin alone, the rate of ABTS oxidation was low; contrarily, in the presence of Xu6 the rate of ABTS oxidation was enhanced significantly. This indicated that the aptamer-hemin complex exhibited peroxidase activity and that the peptide functioned as a peptizyme.

**Molecular recognition**

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The final aim of this study is to create a molecular sequence drawn with a single stroke, that is, a conjugation of peroxidase sequence and a molecular recognition sequence, working like a peroxidase-labeled antibody. As we demonstrated that the complex of the peptide aptamer Xu6 and hemin had peroxidase catalytic activity, we conjugated the molecular recognition sequence FLAG (DYKDDDDK) to Xu6 as a model binding sequence. The FLAG sequence was conjugated to Xu6 via a GGS linker using solid phase synthesis (Table 1). The binding affinity of Xu6-FLAG to hemin was confirmed by UV/Vis and CD spectroscopy (Fig. 2a, Fig. 2b, and Fig. S6), and the association constant of Xu6-FLAG and hemin was determined to be 366 ± 193 μM. The catalytic activity of Xu6-FLAG-hemin complex on an enhanced chemiluminescence (ECL) substrate was also confirmed, by spotting the complex on a membrane and developing chemiluminescence with the ECL substrate, as shown in Fig. 3a. Subsequently, anti-FLAG antibody was spotted on three membranes in dot blots and was probed using the Xu6-FLAG-hemin complex, Xu6-FLAG alone and hemin alone, respectively. Figure 3b shows that only the Xu6-FLAG-hemin complex recognized the anti-FLAG antibody and developed a defined chemiluminescence signal.

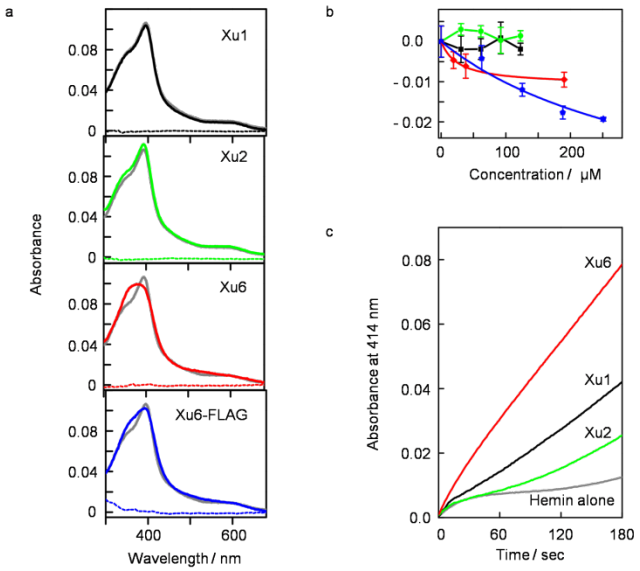


Fig. 2 (a) UV-visible spectra of 2 μM hemin alone (gray) and in the presence of selected peptide aptamers Xu1 (61.4μM), Xu2 (61.4μM), Xu6 (38μM), and Xu6-FLAG (62.7μM) in 40KT buffer, where the contributions of peptides alone (dashed lines) were subtracted: Xu1, black; Xu2, green; Xu6, red; Xu6-FLAG, blue. (b) Peak absorbance change of the UV-visible spectrum of 2 μM hemin in the presence of various concentrations of peptides Xu1, Xu2 and Xu6 and Xu6-FLAG in 40KT buffer, where the contributions of the peptides were subtracted: Xu1, black; Xu2, green; Xu6, red; Xu6-FLAG, blue. (c) The peroxidase activity of different catalyst compositions (hemin alone, gray; Xu1 and hemin, black; Xu2 and hemin, green; and Xu6 and hemin, red) is represented by the time dependency of ABTS oxidation (absorbance at 414 nm). The reaction was carried out in 40KT buffer with peptide, 20 μM; hemin, 0.25 μM; ABTS, 6 mM; $H_2O_2$, 1.8 mM.

Finally, anti-FLAG antibody as well as the negative controls BSA and gelatin were spotted on a membrane in a dot blot, and Fig.3c and 3d shows that the Xu6-FLAG-hemin complex specifically recognized the anti-FLAG antibody and that the complex worked as a peroxidase akin to the horseradish peroxidase commonly used in immunodetection. Furthermore, the catalytic activities of the Xu6-hemin complex and HRP were compared by determining the rates of ABTS oxidation (Fig. 3e).

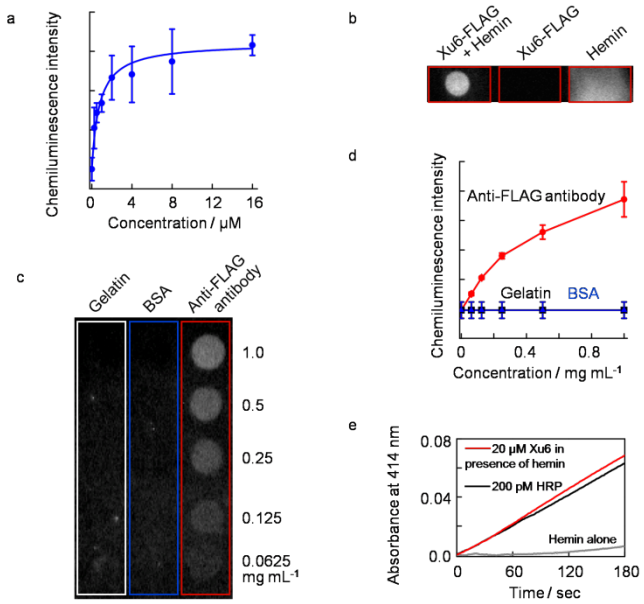


Fig. 3 (a) Confirmation of catalytic activity of Xu6-FLAGand hemin complex on ECL substrate on PVDF membrane. Two microliters of various concentrations of Xu6-FLAG and 0.25 μM hemin complexes in milli-Q water were placed on the membrane, and the chemiluminescence intensity was detected using the ECL kit. (b) Confirmation of the molecular recognition and catalytic activity of Xu6-FLAG-hemin complex. One hundred microliters each of 100 μM Xu6-FLAG with2 μM hemin, 100 μM Xu6-FLAG alone, and 2 μM hemin alone, were used for the detection of anti-FLAG antibody in 3 dot blots, respectively. Two microliters of 1 mg $mL^{-1}$ anti-FLAG antibody in PBS buffer was used for each spot. (c) Molecular recognition study of Xu6-FLAG. Six hundred microliters of 100 μM Xu6-FLAG and 2 μM hemin complex was used for the detection of anti-FLAG antibody in a dot blot; 1 mg $mL^{-1}$ of anti-FLAG antibody, BSA and gelatine were diluted in PBS buffer with dilution ratios of 1:1, 1:2, 1:4, 1:8 and 1:16, respectively, and 2 μL of each solution was used for each drop. (d) Integrated intensity of the dot blot chemiluminescence image of (c) for various concentrations of anti-FLAG antibody (red), BSA (blue) and gelatin (black). (e) The peroxidase activity of different catalyst compositions (hemin alone, gray; Xu6 and hemin, red; HRP, black) is represented by the rate of ABTS oxidation (absorbance at 414 nm). The reaction was carried out in 40KT buffer with hemin, 0.25 μM; Xu6, 20 μM; HRP, 200 pM; ABTS, 6 mM; $H_2O_2$, 1.8 mM.

## Conclusion

A dual functionalized peptide chain that contains a molecular recognition sequence and an *in vitro* selected catalytic

sequence was successfully designed and demonstrated. A complex was formed with hemin, and the complex successfully recognized the analyte and simultaneously worked as a peroxidase. This study demonstrated a successful strategy to create dual functionalized peptide chains for various immunoassays.

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