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Preparation of ferrocene-functionalized gold nanoparticles by primer extension reaction on the particle surface

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ABSTRACT

DNA molecules possessing multiple ferrocene (Fc) molecules as a redox active probe were prepared by the primer extension (PEX) reaction using a 2′-deoxyuridine-5′-triphosphate derivative in which Fc was connected to the C5-position of the uridine by a diethylene glycol linker. Gold nanoparticles (AuNP) covered with DNA possessing the Fc molecules were prepared by the PEX reaction on the surface. The AuNP–FcDNA conjugates exhibit a detectable electrochemical signal due to the Fc molecules. Possible application of the PEX reaction on AuNP is demonstrated for the detection of a single nucleotide mutation in the target DNA.

Nucleic acid derivatives possessing functional molecules at various positions have been designed and prepared for biochemical and medicinal applications, such as the fluorescent and electrochemical detections of DNA.[1] Many functional nucleic acid derivatives have been synthesized by a phosphoramidite method using an automated DNA synthesizer.[1] Recently, an enzymatic method based on the DNA polymerase reactions has proven to be effective, facile, and applicable for the multiple incorporation of functional molecules into DNA.[2] The enzymatic method allows us to control the number and arrangement of functional molecules, such as fluorophore or redox active molecules on DNA.[2] Fojta and Hocek have reported a method for the synthesis of base-modified deoxyribonucleoside triphosphates by an aqueous cross-coupling reaction, and have successfully incorporated them into DNA.[3]

The multiple-labeling of DNA has some advantages in the detection of DNA and biomolecules because of the possible generation of strong signals derived from the fluorescence and redox labels.[4–9] Electrochemical sensors have become promising because they offer a more convenient and cost-effective method for the detection of DNA and biomolecule when compared to optical devices.[10–12] In fact, unique electrochemical sensors based on DNA probes and aptamers targeting various kinds of substances from small molecules, metal ions, and oligonucleotides to large proteins have been developed.[3,13]

In this paper, we report the enzymatic incorporation of ferrocene (Fc) molecules into DNA by the primer extension (PEX) reaction using a deoxyuridine triphosphate (dUTP) labeled with Fc as a redox reporter. We prepared Fc-modified deoxyuridine triphosphate (Fc-dUTP) using an aqueous cross-coupling reaction between 5-iodo-dUTP and Fc-derivatives having an ethynyl group. The important finding is that the Fc-dUTP using a longer ethylene glycol linker efficiently affords the full-length product (FcDNA) in the PEX reaction. Moreover, the PEX reaction or the incorporation of Fc molecules into DNA can proceed on gold nanoparticles (AuNPs). We demonstrated that the PEX reaction on AuNPs is applicable for the electrical detection of a single nucleotide mutation in the target DNA.

2′-Deoxyuridine triphosphates possessing ferrocene as a redox active probe at the C5-position of the pyrimidine ring were prepared following the method developed by Hocek et al.[3] Two derivatives (**FU1** and **FU2**) were prepared by the Sonogashira cross-coupling reaction between 5-iododeoxyudirine triphosphate and ferrocene derivatives bearing an ethynyl group (Fig. 1A). The modification at the C5-position of the pyrimidine base is known to be useful in the design of the triphosphate analogs with a high tolerance to the functional groups in the polymerase reactions.[3] In this study, the ferrocene ring was attached through the propargyl group to the C5-position of uracil base (**FU1**). In **FU2**, a diethyleneglycol linker was inserted as a spacer between the ethynyl group and the ferrocene ring.

Two kinds of thermostable polymerases categorized to family B (KOD Dash and vent (exo-)), showing a high tolerance to a wide

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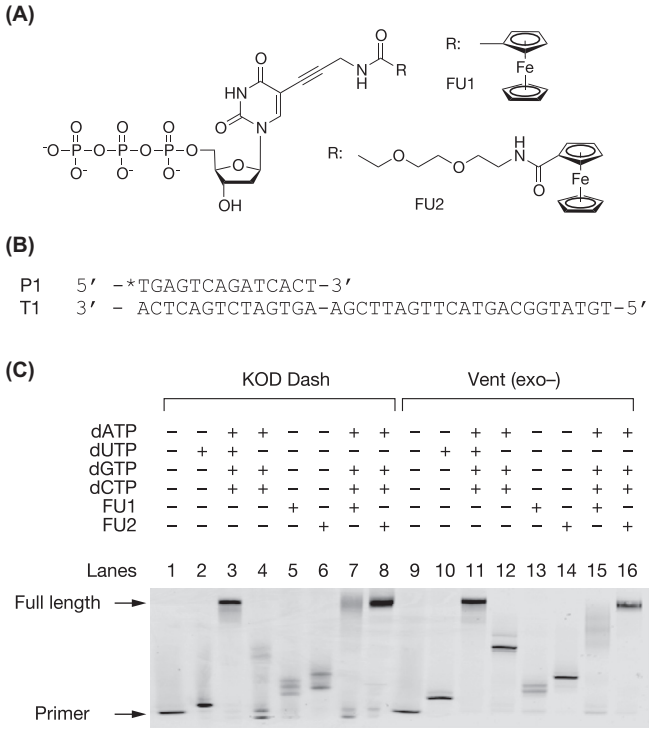

Figure 1. (A) Structures of 2′-deoxyuridine triphosphate possessing ferrocene (Fc) as a redox active probe at the C5-position of the pyrimidine ring (**FU1** and **FU2**). (B) Sequence of primer (**P1**) and template (**T1**) used for the primer extension (PEX) reaction. **P1** was labeled with a fluorescein at the 5′-terminus. (indicated by asterisk). (C) A 15% denaturing PAGE of the primer extension products obtained using the Fc-labeled dUTP and polymerase KOD Dash (lanes 1–8) or Vent (exo-) (lanes 9–16).

range of triphosphate analogs with modification at the C5-position,[7] were used to investigate the primer extension (PEX) reaction using the **FU1** or **FU2**. The single-nucleotide extension and full-length extension reaction were investigated by denaturing polyacrylamide gel electrophoresis (PAGE) (Fig. 1B and C). A 14-mer primer (**P1**) labeled with a fluorescein and 34-mer DNA template (**T1**) were hybridized, and then subjected to the PEX reaction using **FU1** or **FU2** as a substrate instead of dUTP for the polymerase reaction. When the reaction mixture contained only **FU1** or **FU2** (single nucleotide extension), electrophoretic bands corresponding to the **P1** disappeared after the reaction and a slow-migrating band appeared (lanes 5 and 6 for KOD Dash, and lanes 13 and 14 for Vent (exo-)), indicating the incorporation of **FU1** and **FU2** by these polymerases. A full-length PEX product was obtained for **FU2** (lane 8 for KOD Dash, lane 16 for Vent (exo-)), whereas **FU1** did not afford the full-length product efficiently. This is probably due to the steric hindrance of the bulky ferrocene molecule that is close to the recognition site in the polymerase extension reactions. In contrast, the Fc of **FU2** connected by the ethylene glycol linker did not interfere with the polymerase reaction, leading to an efficient enzymatic incorporation. A similar positive effect of the ethylene glycol linker providing an efficient incorporation has also been reported.[14]

The preparation of AuNP/DNA conjugates possessing the Fc molecules on the surface by the PEX reaction are schematically depicted in Figure 2A. **FU2** was used for this experiment because **FU2** had shown a better incorporation efficiency than **FU1**. The primer was immobilized on the surface of AuNP and hybridized with the template DNA, and then the PEX reaction was carried out using **FU2** to incorporate the Fc molecules into DNA on the surface.[15] AuNP modified with the primer DNA (**P2**) and diluter DNA (**D**) (1:1) was prepared according to the standard protocol using

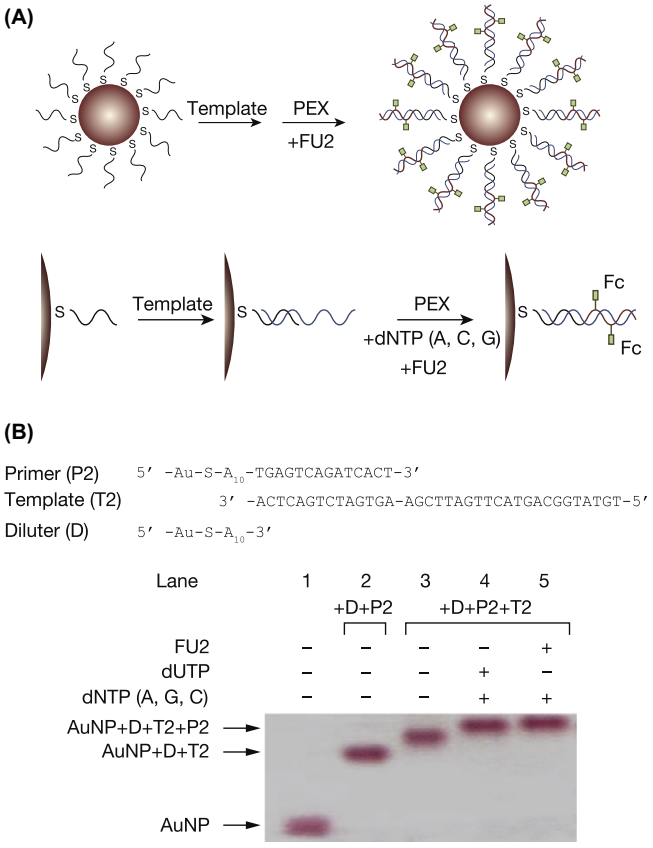

Figure 2. (A) Schematic illustration of enzymatic approach to prepare AuNP modified with multiple Fc molecules on the surface by the PEX reaction. (B) Non-denaturing 3% agarose gel electrophoresis of AuNP subjected to the PEX reaction using **FU2** as a substrate of the polymerase reaction. The PEX reaction was carried out with a primer (**P2**) and template (**T2**) immobilized on AuNP (20 nm) using Vent (exo-). The reaction was carried out at 37 °C for 30 min. Lanes 1–3: unmodified AuNP, AuNP/**D**/**P2**, AuNP/**D**/**P2**/**T2**. Lanes 4 and 5: AuNP conjugates after the PEX reaction using dUTP (lane 4) and **FU2** (lane 5).

oligonucleotides with a thiol group.[16] Surface coverage of AuNP with **P2** was reduced by adding a short 10-mer dA (**D**) in order to facilitate the hybridization between the primer and the template, reduce the steric hindrance and enhance the polymerase activity.[15] A 36-mer template DNA (**T2**) was then hybridized with **P2** on the surface.

The PEX reaction on AuNP was analyzed by agarose gel electrophoresis (Fig. 2B). All electrophoretic bands were visualized by the strong red color of the AuNP. The surface-modified AuNP with **D**/**P2** (lane 2) ran much slower than the unmodified AuNP (lane 1). After hybridization with **T2**, the AuNP conjugates showed decreased mobility. The PEX reaction with Vent (exo-) polymerase on the surface of AuNP was carried out by dUTP (lane 4) or **FU2** (lane 5). After the PEX reaction, both AuNP conjugates showed slower bands compared to the unreacted AuNP conjugates (lane 3). This difference in the electrophoretic mobility means that the extension reaction on the surface occurred. The slightly slower band of the AuNP conjugates by **FU2** than that by dUTP may correspond to the multiple incorporation of Fc molecules causing an increased molecular size.

The electrochemical response of the AuNP/DNA conjugates possessing the Fc molecules prepared by the PEX reaction was investigated (Fig. 3A).[17] The surface of 20 nm AuNP is covered with the capture (**C1**), primer (**P3**), and diluter DNA (**D**) (1:1:1), and then hybridized with the template DNA (**T3**). Template DNAs with A or T next to the starting point of the extension reaction were used. After hybridization, the PEX reaction was carried out using the **FU2** as

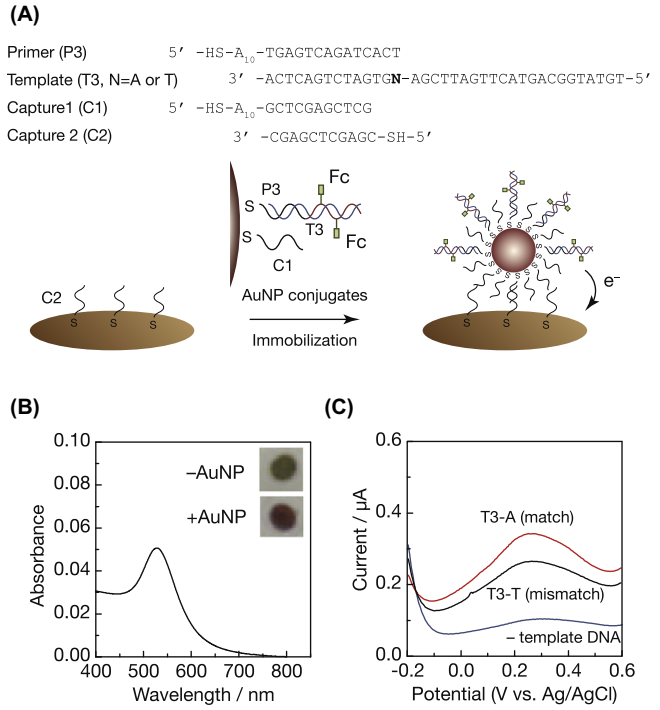

Figure 3. (A) Immobilization of AuNP/DNA conjugates possessing the Fc molecules by the PEX reaction on the electrode. Sequences of primer (**P3**), template (**T3**), capture DNAs (**C1**) and (**C2**). The letter 'N' in **T3** represents the A or T base. The gold electrode was modified with **C2**, and the AuNP conjugates after the PEX reaction were immobilized on the surface via hybridization between **C1** and **C2**. (B) UV/vis absorption spectrum of AuNP/DNA conjugates recovered from the electrode through dehybridization in hot water. Inset in the figure shows pictures of the electrode before and after immobilization of the AuNP conjugates. (C) Differential pulse voltammetry (DPV) response of the electrode modified with AuNP conjugates prepared using two template DNAs (**T3-A**, **T3-T**) or without template DNA.

the substrate for Vent (exo-). We assumed that the Fc molecules could be efficiently incorporated when N of the template DNA is A (**T3-A**), whereas the PEX reaction is inhibited in **T3-T** due to the mismatch base pair. After the PEX reaction, the AuNP conjugates were immobilized on the gold electrode through the hybridization of the capture **C1** on AuNP with **C2** on the electrode. Immobilization of AuNP on the electrode was confirmed by the color change of the electrode surface originated from the red color of the AuNP particles. (Fig. 3B, inset). The AuNP conjugates can be released from the surface by immersing the electrode in hot water. The resulting solution was analyzed by UV/vis absorption measurements. The plasmon absorption band with a maximum around 520 nm was observed (Fig. 3B). This is another indication for the surface immobilization of the AuNP/DNA.

Electrochemical responses of the AuNP/DNA conjugates immobilized on the electrode are shown in Figure 3C. As expected, the electrochemical signals of the differential pulse voltammetry (DPV) for the AuNP/DNA conjugates prepared using **T3-A** were observed at ~0.3 V versus Ag/AgCl, which contributed to the oxidation peak of the Fc molecules on the surface.[18] In contrast, little electrochemical signal was observed for the AuNP/DNA prepared without using the template DNA. These data indicate that the observed electrochemical signal for the AuNP/**FU2**-DNA prepared using **T3-A** is resulted from the oxidation of the ferrocene (Fc) molecules. When **T3-T** was used as a template, the suppressed electrochemical signal of Fc was observed. This signal reduction is caused from the poor incorporation of the redox molecules by the T–T mismatch base pair. The detectable difference in the electrochemical measurements between the matched and mismatched template DNA sequences suggests that the present approach may be applicable to identify the single nucleotide variation in DNA. Electrochemical detection of nucleic acid sequences based on PEX reactions has been reported.[3,12,19] Our present approach contains the combined use of a DNA polymerase reaction on nanoparticles and immobilization of redox-modified DNA-nanoparticles on an electrode surface. Therefore, the method presented herein is useful for multiple incorporation redox molecules into DNA on an electrode that should lead to a highly sensitive electrochemical detection of DNA.

We have described the enzymatic incorporation of ferrocene (Fc) molecules into DNA using Fc-modified deoxyuridine triphosphates (Fc-dUTP) as a substrate for the DNA polymerase. The Fc-dUTP using a longer ethylene glycol linker efficiently afforded the full-length product (FcDNA) in the primer extension (PEX) reaction. It was also shown that the PEX reaction or the incorporation of Fc molecules into DNA can proceed on gold nanoparticles (AuNP). Electrochemical experiments showed that the AuNP–FcDNA conjugates exhibit an electrochemical signal from the Fc molecules. The electrochemical signal was reduced by a single base mismatch in DNA, indicating the potential utility of the present method for the detection of a single nucleotide mutation in DNA.

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Supplementary data

Supplementary data (synthesis of the triphosphate derivatives, and experiment details) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.062.

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