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Communications

Preparation of fluorescent nucleic acids generating unique emission by primer extension reaction using pyrene-labeled deoxyuridine triphosphate derivatives

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ABSTRACT: Fluorescent nucleic acids were prepared utilizing the polymerase extension (PEX) reaction to incorporate fluorescent molecules. 2'-Deoxyuridine triphosphate (dUTP) derivatives possessing pyrene molecules as fluorophores were synthesized using the aqueous-phase Sonogashira coupling between 5-Iodo-dUTP and acetylene-linked pyrene molecules. The incorporation of the pyrene (Py)-labeled deoxyuridine triphosphates (PyU) into DNA by polymerase was evaluated by polyacrylamide gel electrophoresis, demonstrating that the PyU can work as a good substrate for the PEX reaction. The fluorescent properties of the functionalized DNA prepared by the PEX reaction were characterized by steady-state fluorescence measurements. The Py-conjugated DNA showed typical emission spectra of the pyrene, and the DNA with two pyrene molecules connected to each other by a diethylene glycol linker exhibited a broadened emission attributed to the electronic interaction between the Py molecules.

Nucleic acids modified with functional groups/molecules are of significant interest as potential applications in chemical biology, diagnostic tools or nanotechnology and material science.[1-3] To expand their applications as versatile tools, various kinds of oligodeoxynucleotide modified with functional molecules have been prepared by a general synthetic approach using the corresponding phosphoroamidite units during solid-phase DNA synthesis and the combination with post-synthetic oligonucleotides modification.[4,5] As an alternative approach to the conventional DNA synthesis for the preparation of the functionalized oligonucleotides, an enzymatic method using the polymerase extension (PEX) reaction has attracted much attention because of the facile preparation of longer oligonucleotides from DNA templates and multiple incorporation of functional groups/molecules into DNA based on the sequence of the templates.[6,7]

Certain kinds of deoxynucleotide triphosphate (dNTP) derivatives possessing a fluorescent reporter group at an appropriate position on the nucleobase can work as effective substrates for the enzymatic incorporation by polymerase and are used as fluorescent probes for the detection of the hybridization and amplification of DNA.[3,8-12] Pyrene fluorophores with unique fluorescent properties, capable of generating an excited dimer with a characteristic green light, were conjugated to oligonucleotides for the structural study of the folding and development of single nucleotide polymorphisms (SNPs).[13-1521] In this study, we demonstrated the preparation of the deoxyuridine triphosphate derivatives labelled with the pyrene (Py) fluorophore through an ethylene glycol linker, and evaluation of the enzymatic incorporation of the triphosphate by DNA polymerase and the emission properties of the Py-incorporated DNA.

Multiple incorporation of the pyrene molecules into DNA by the polymerase extension (PEX) reaction is schematically shown in Figure 1. We have chosen pyrene (Py) as the fluorescent molecule because pyrene is known to show a strong emission and unique emissive behavior depending on the local environment and electronic interaction between the pyrene molecules.[16] The PEX reaction using pyrene-modified deoxyuridine triphosphates (Py-dUTP) instead of unmodified dUTP can allow us to introduce the pyrene molecules into DNA. The Py-conjugated dUTP were synthesized by the aqueous-phase Sonogashira coupling reaction between 5-iodo-dUTP and pyrene derivatives with an acetylene unit,[17,18] as shown in Figure 1b. The acetylene unit was connected to the pyrene molecules through a relatively long ethylene glycol linker to minimize the steric hindrance of the pyrene during the polymerase reaction, and the pyrene was attached at the 5-position of the uridine base through the linker because several polymerases showed a high tolerance to the presence of the functional group at this position. The 5-I-dUTP was prepared according to a previous paper.[19] After the reaction, the triphosphate (**PyU1** and **PyU2**) was purified by reverse-phase HPLC and characterized by mass spectrometry.

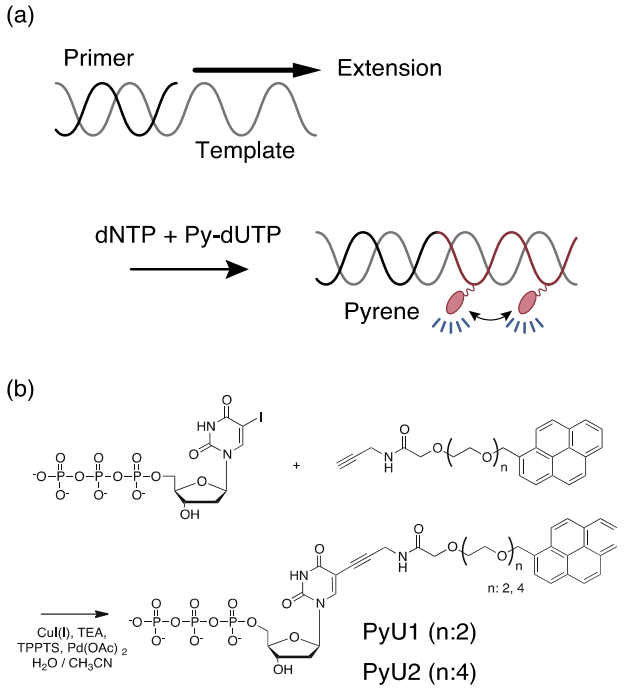

Figure 1. (a) Schematic illustration of the multiple incorporation of pyrene (Py) molecules into DNA by the primer extension (PEX) reaction. The Py molecules can be incorporated during the PEX reaction when the pyrene-labeled deoxyuridine triphosphate (Py-dUTP) was used instead of dTTP or dUTP. (b) Synthesis of the Py-labeled deoxyuridine triphosphate by aqueous-phase Sonogashira coupling reaction between 5-Iodo-dUTP and pyrene with an ethynyl group.

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P1 5′ -Fl-TGAGTCAGATCACT
T1 3′ -ACTCAGTCTAGTGA-**A**GCTT**A**GTTC**A**TG**A**CGGT**A**TGT-5′

P2 5′ -Fl-AGAGAGAGAGAAAA
T2 3′ -TCTCTCTCTCTTTT-**A**TT**A**TT-5′

Scheme 1. DNA sequences used in this study. Primers (**P1** and **P2**) were modified with a fluorescein (**Fl**) at 5'-terminus for PAGE analysis. Incorporation sites corresponding to the position of adenines in the templates are shown in bold.

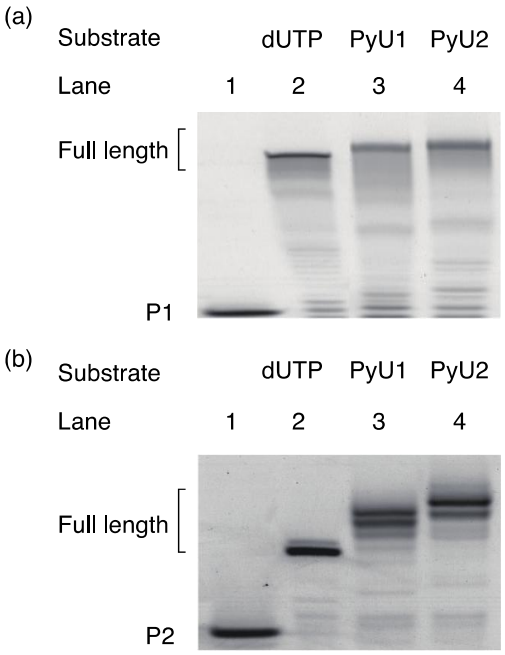

Figure 2. PAGE analysis of primer extension (PEX) reaction with KOD dash polymerase using the **PyU1** and **PyU2** instead of dUTP in the presence of three other triphosphates (dGTP, dATP, dCTP). (a) **P1**/**T1,** lane 1: only primer, lane 2: dUTP, lane 3: PyU1, lane 4: PyU2. (b) **P2**/**T2,** lane 1: only primer, lane 2: dUTP+dATP, lane 3: **PyU1**+dATP, lane 4: **PyU2**+dATP.

Incorporation of pyrene-labeled triphosphate derivatives by polymerase extension into DNA using **PyU1** and **PyU2** were investigated by denaturing polyacrylamide gel electrophoresis (PAGE). DNA sequences used for PEX reaction are shown in Scheme 1. The primer strands, **P1** and **P2**, were modified with fluorescein for visualization after PAGE. Templates **T1** and **T2** were designed so as not to have a continuous adenine sequence because the incorporation efficiency of the modified dNTP is known to be inefficient.[20] The PEX reaction was carried out with the KOD dash polymerase because this polymerase showed a high incorporation efficiency of the triphosphate derivatives with a functional group at the 5-position of the pyrimidine base. Figure 2 shows the results of the PAGE analysis after the enzymatic reaction. The electrophoresis bands close to the bands of the full-length product in dUTP were observed when either **PyU1** or **PyU2** was used as a substrate instead of dUTP, indicating that both **PyU1** and **PyU2** were successfully incorporated into the DNA during the PEX reaction. The difference in the linker length did not affect the incorporation efficiency. The slightly slow migration bands of **PyU1** and **PyU2** compared to the band of dUTP also indicated the incorporation of the pyrene molecule into DNA because migration in the gel depended on the molcular size. Similar results were obtained in the case of **P2**/**T2**. Products bands obtained using either **PyU1** or **PyU2** corresponding to the full-length product migrated slowly compared to the product of dUTP, which means that the **PyU1** and **PyU2** were efficiently incorporated. These results clearly showed that the dUTP derivatives possessing the pyrene molecule at the 5-position of the base through the ethylene glycol linker can work as a good substrate for the PEX reaction.

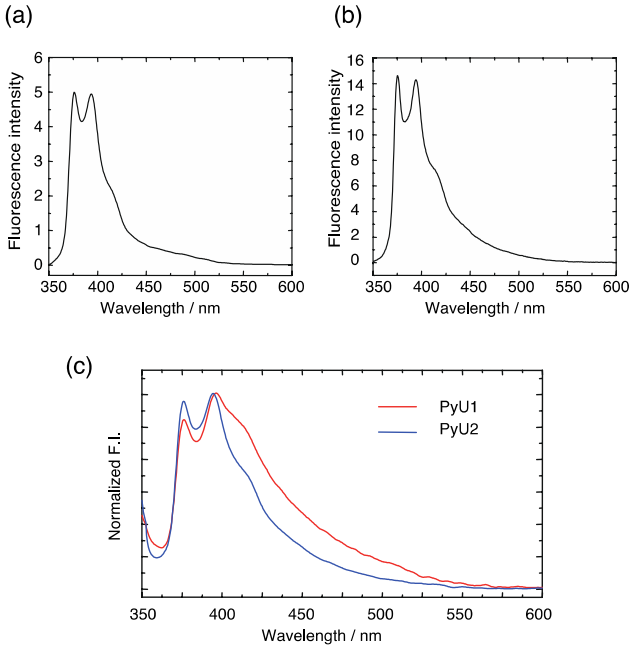

Figure 3. Fluorescence spectra of pyrene-labeled triphosphates, (a) **PyU1** and (b) **PyU2**, and (c) DNA oligonucleotides possessing the pyrene molecules prepared by the PEX reaction with KOD Dash polymerase on **P2**/**T2** in an Na phosphate buffer (pH 7, 100 mM NaCl). **P2** without a fluorescein was used for the PEX reaction. The excitation wavelength was 340 nm.

The emission properties of the pyrene-incorporated DNA were investigated based on fluorescence measurements in Na phosphate buffer (pH 7.0, 100 mM NaCl) (Figure 3). The triphosphate derivative (**PyU1** and **PyU2**) in an aqueous solution showed a typical emission spectra of the pyrene molecule with peaks at 380 and 400 nm (Figuress 3a and b). The Py-incorporated DNA duplex was prepared by the PEX reaction using the **P2**/**T2** and **PyU1** or **PyU2**, and the florescence of the products were measured after the purification of the reaction solution using DNA purification kits (Figure 3c). Both products showed emission spectra similar to that of **PyU1** and **PyU2**, demonstrating that the Py molecules were incorporated into the DNA by the PEX reaction and the Py molecules in DNA were emissive. In the case of **PyU2**, the emission spectrum of the products obtained with **P2**/**T2** showed a spectrum almost similar to that of the triphosphate derivatives, while the product of **PyU1** showed much broader emission spectrum. This implied that the electronic interaction between two pyrene molecules affected the excited state of the pyrene. It is expected that the pyrene molecules in the products of **PyU1** and **PyU2** had conformational freedom due to the long ethylene glycol linker. A shorter linker of the **PyU1** than **PyU2** may increase the probability of contact with another pyrene, contributing to the increase in the intermolecular interaction and the broader emission.

In this study, we synthesized the deoxyuridine triphosphates with a pyrene that was connected to the 5-position of the uridine through an ethylene glycol linker, and showed that the triphosphate

derivatives can be efficiently incorporated into the DNA by the PEX reaction. Fluorescent measurements showed that the DNA products after the enzymatic reaction displayed a typical emission spectrum of the pyrene molecule, thus the fluorescent labeling of the oligonucleotide with the pyrene by the PEX reaction was achieved. In addition, the DNA products with two pyrenes that are connected with a diethylene glycol linker showed a somewhat broadened emission longer than 400 nm, indicating the electronic interaction between these pyrene molecules. Our study showed that the deoxyuridine triphosphate with the pyrene linked with an ethylene glycol linker can be an efficient substrate for the polymerase reaction, allowing us to prepare fluorescent nucleic acids that can be used as for the fluorescent probes and sensors.

KEYWORDS: DNA, fluorescence, pyrene, polymerase extension reaction.

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