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Sequence-Selective Single-Molecule Alkylation with a Pyrrole–Imidazole Polyamide Visualized in a DNA Nanoscaffold

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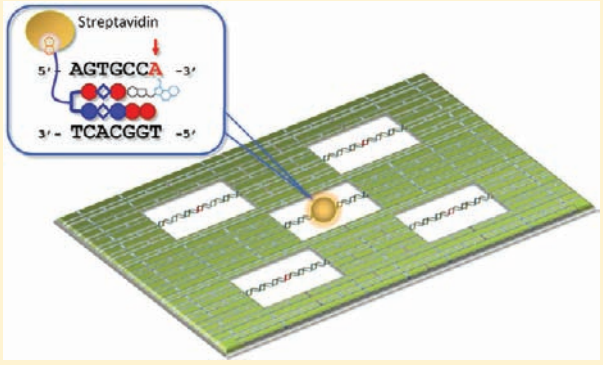
Supporting Information

ABSTRACT: We demonstrate a novel strategy for visualizing sequence-selective alkylation of target double-stranded DNA (dsDNA) using a synthetic pyrrole–imidazole (PI) polyamide in a designed DNA origami scaffold. Doubly functionalized PI polyamide was designed by introduction of an alkylating agent 1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (*seco*-CBI) and biotin for sequence-selective alkylation at the target sequence and subsequent streptavidin labeling, respectively. Selective alkylation of the target site in the substrate DNA was observed by analysis using sequencing gel electrophoresis. For the single-molecule observation of the alkylation by functionalized PI polyamide using atomic force microscopy (AFM), the target position in the dsDNA (~200 base pairs) was alkylated and then visualized by labeling with streptavidin. Newly designed DNA origami scaffold named "five-well DNA frame" carrying five different dsDNA sequences in its cavities was used for the detailed analysis of the sequence-selectivity and alkylation. The 64-mer dsDNAs were introduced to five individual wells, in which target sequence AGTXCCA/TGGYACT (XY = AT, TA, GC, CG) was employed as fully matched (X = G) and one-base mismatched (X = A, T, C) sequences. The fully matched sequence was alkylated with 88% selectivity over other mismatched sequences. In addition, the PI polyamide failed to attach to the target sequence lacking the alkylation site after washing and streptavidin treatment. Therefore, the PI polyamide discriminated the one mismatched nucleotide at the single-molecule level, and alkylation anchored the PI polyamide to the target dsDNA.



INTRODUCTION

Single-molecule visualization is a practical approach for investigating recognition of biomolecules and reaction with target DNA sequences.[1] Atomic force microscopy (AFM) enables observation of biomolecules at nanoscale spatial resolutions; however, an observation scaffold is required for accurate analysis of the precise interactions and reactions.[1] DNA origami has recently been developed for construction of a wide variety of multidimensional nanostructures that can be used as scaffolds for incorporation of various functionalities at specific positions and for analysis of single-molecule interactions and chemical reactions.[1−8] We recently developed an AFM-based analysis system using a DNA origami scaffold to incorporate various double-stranded DNA (dsDNA) substrates.[9] Using a frame-like DNA origami scaffold, multiple dsDNAs can be applied onto the cavity for studying enzymatic reactions and DNA structural changes at the single-molecule level.[9]

A pyrrole–imidazole (PI) polyamide is one of the well-recognized sequence-specific DNA binders and its DNA recognition sequences can be systematically designed.[10] In addition, modified PI polyamides with target-orientated functionalities are employed in various fields, including cell biology and nanotechnology.[11,12] For the biological application, DNA alkylating molecules have been developed as antitumor agents, which form covalent bonds with nucleotide bases at specific positions.[13] By introducing them to a PI polyamide, we have investigated a PI polyamide–alkylating agent conjugate, which can be used as a sequence-selective synthetic DNA alkylator applicable to gene regulators.[14]

In this study, we demonstrate single-molecule analysis of sequence-selective recognition and alkylation of a PI polyamide–alkylating agent conjugate in the DNA origami scaffold. For this purpose, we designed and synthesized a doubly

Received: October 2, 2011
Published: February 9, 2012

dx.doi.org/10.1021/ja209023u | *J. Am. Chem. Soc.* 2012, 134, 4654–4660

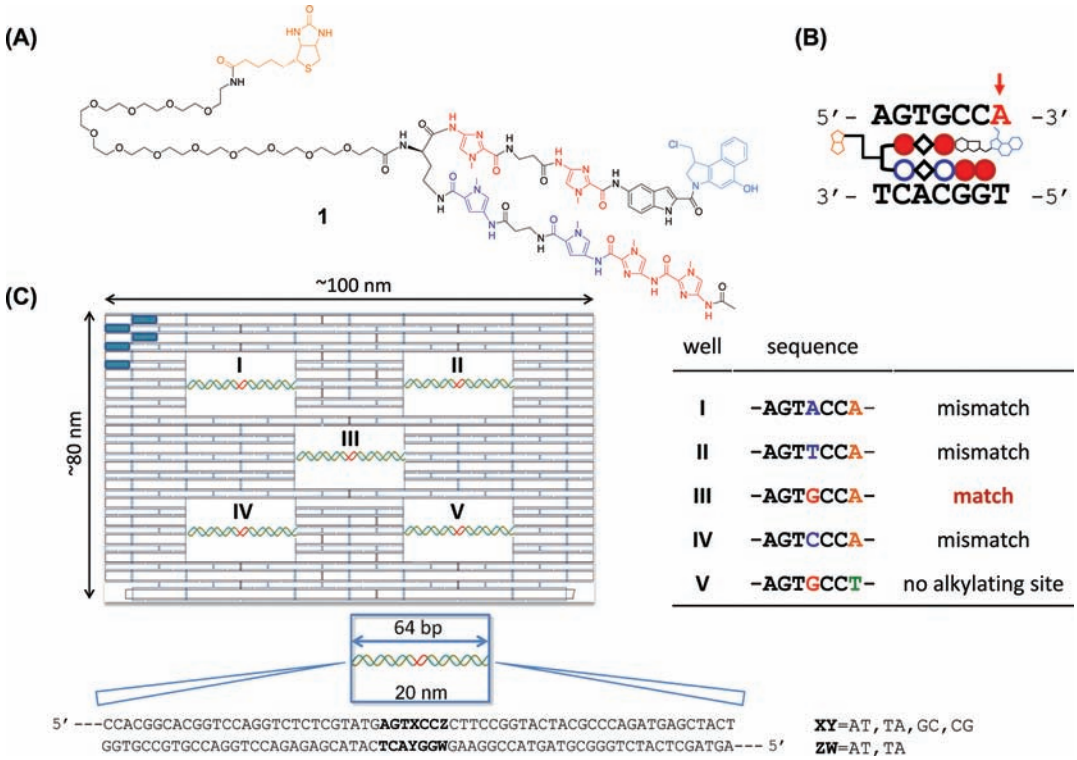


Figure 1. Doubly functionalized PI polyamide and the five-well DNA frame used in the experiment. (A) Chemical structure of biotinylated PI polyamide–*seco*-CBI conjugate **1**. (B) The target sequence for the conjugate **1**. A red arrow indicates the target adenine for alkylation (N3 position of adenine). (C) Structure of the five-well DNA frame (5WF). Five different 64-mer dsDNAs were incorporated to the 5WF scaffold. Five hairpin DNAs were introduced at the left top corner of 5WF as the orientation marker (blue rectangles). The sequence of the incorporated 64-mer dsDNA is presented at the bottom. The five dsDNA sequences incorporated into the corresponding wells are presented at the right.

functionalized PI polyamide possessing a 1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (*seco*-CBI) unit for DNA alkylation and a biotin unit for single-molecule observation (Figure 1A). The designed PI polyamide recognizes the six-base pair sequence 5′-AGTGCC-3′ and alkylates the N3 position of the adjacent adenine at the 3′-side (Figure 1B). We also designed a DNA scaffold with five rectangular cavities inside, which we named a "five-well DNA frame" (Figure 1C). We placed five different dsDNA sequences containing a recognition sequence of PI polyamide and an alkylating site onto the five-well DNA frame (5WF) and performed the five different DNA alkylating reactions at the same time. The sequence-selectivity and the requirement of alkylation were analyzed at the single-molecule level using the 5WF scaffold and AFM analysis.

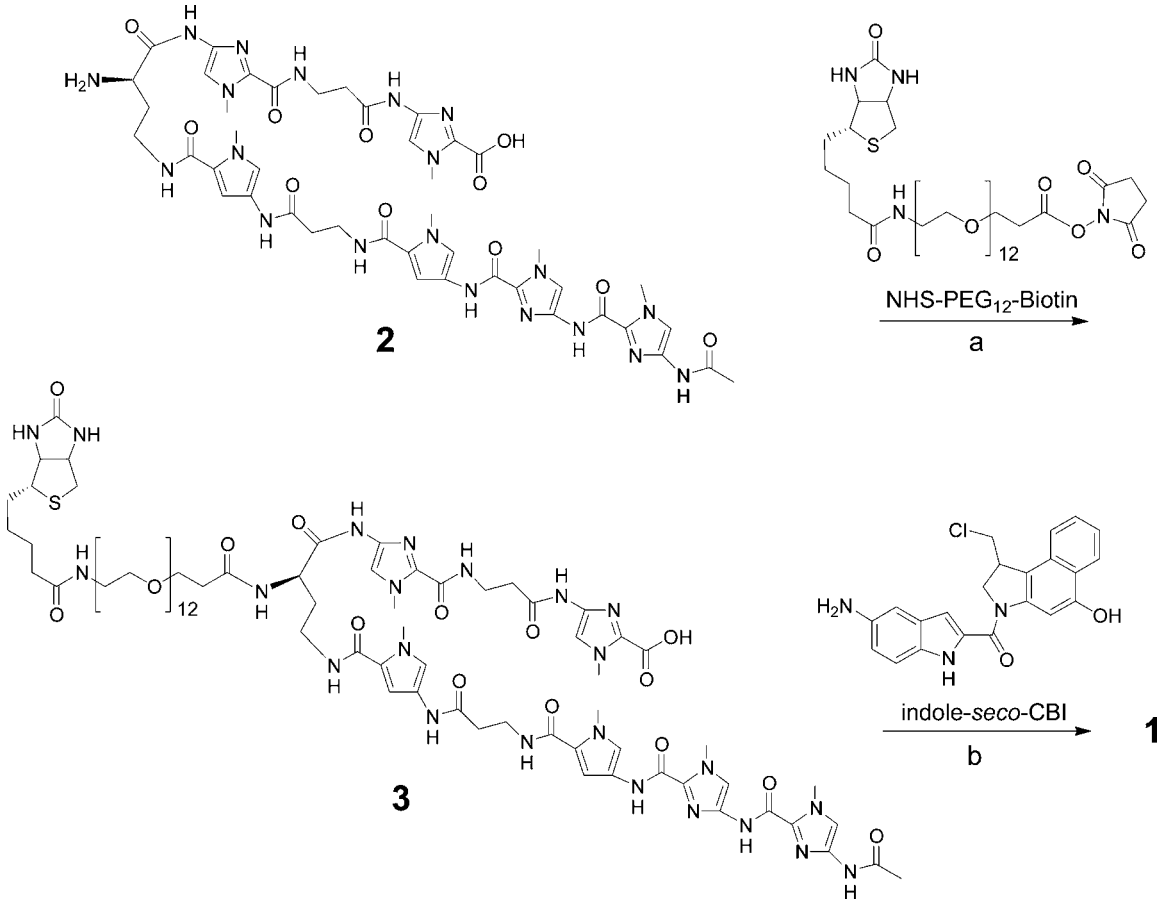
RESULTS AND DISCUSSION

Preparation of the PI Polyamide Conjugate. We designed a doubly functionalized PI polyamide which has an alkylating agent *seco*-CBI at the C-terminal end and a biotin unit at the hairpin-turn (Figure 1B). The core moiety of the PI polyamide contains ImImPy-β-Py-$(R)^{H2N}\gamma$-Im-β-Im, where Im, Py, β, and γ represent *N*-methyl imidazole, *N*-methyl pyrrole, β-alanine, and γ-aminobutyric acid, respectively. This PI polyamide recognizes 5′-WGWGCC-3′ (W = A or T). The synthesis of conjugate **1** is shown in Scheme 1. (*R*)-2,4-Diaminobutyric acid ($(R)^{H2N}\gamma$) was used for a hairpin-turn component because $(R)^{H2N}\gamma$ does not have steric effects as compared with (*S*)-2,4-diaminobutyric acid.[15] The PI polyamide was synthesized using Fmoc-based solid-phase synthesis.[16] Cleavage from resin and deprotection of the Boc group were carried out using 1 mL of TFA/water/triisopropylsilane mixture to produce compound **2**. Coupling of crude **2** with a Biotin-PEG_{12}-*N*-hydroxysuccinimide ester (NHS) gave compound **3** possessing a biotin unit. Subsequently, the amino-indole–*seco*-CBI dimer was coupled with carboxylic acid **3** in dimethylformamide (DMF) using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and $^{i}Pr_2NEt$ to produce the desired polyamide–indole–*seco*-CBI conjugate **1** with a biotin unit. After the purification using a reverse-phase HPLC, the conjugate **1** was identified by electron spray ionization time-of-flight mass spectrometry (ESI-TOF-MS).

Analysis of Sequence-Selective Alkylation of the PI Polyamide Conjugate Using Sequencing Gel Electrophoresis. Alkylating activity of PI polyamide conjugate **1** was analyzed by polyacrylamide gel electrophoresis (PAGE) using sequencing gel (Figure 2). The target site to be alkylated in a 208 bp DNA fragment is shown in Figure 2A. Alkylation of the DNA fragment containing the target sequence was carried out at 23 °C for 24 h, and the reaction was quenched by addition of calf thymus DNA. Then, the samples were heated at 95 °C for 20 min under neutral conditions, which induced the strand cleavage of the alkylated site. Alkylated sites of purine N3 in the DNA can be cleaved by this treatment, and the cleavage bands appear on the gel.[17] After the incubation and following treatment, the sequencing PAGE was performed. As shown in Figure 2B, single cleavage band appeared on the gel, and the sequence of the cleavage site was 5′-AGTGCCA-3′. The result shows that the PI polyamide conjugate **1** recognizes the target site 5′-AGTGCCA-3′ in the substrate DNA and alkylated the adenine at the 3′-end (underlined). In addition, the alkylation

dx.doi.org/10.1021/ja209023u | *J. Am. Chem. Soc.* 2012, 134, 4654–4660

Scheme 1. Synthetic Scheme for the Preparation of Conjugate 1[a]



[a](a) *N*-Hydroxysuccinimde ester (NHS)-PEG_{12}-Biotin, $^{i}Pr_2NEt$, DMF; (b) indole−*seco*-CBI, $^{i}Pr_2NEt$, DMF.

proceeded in a concentration-dependent manner (Figure 2C). These results show that selective alkylation occurred at the single site as designed using this PI polyamide conjugate. In addition, the biotin unit did not inhibit the recognition and alkylation ability of the PI polyamide.

AFM Analysis of dsDNA Alkylation by PI Polyamide Conjugate 1. Alkylation of the dsDNA containing the target sequence was analyzed using AFM. The DNA fragment (208 bp), which was used in the sequencing PAGE analysis and contains the target sequence, was incubated with conjugate **1** at 25 °C for 24 h. After the incubation, the resulting product was labeled with streptavidin, deposited on a mica surface, and imaged in a buffer solution using AFM. Streptavidin bound to the DNA fragment was observed (Figure 3A). Analysis of the binding positions and distribution showed that the streptavidin bound to the 112 ± 4 bp position, which was close to the expected alkylation site (115 bp) (Figure 3B,C). The result showed that the binding can be visualized at the single-molecule level using streptavidin labeling, and the PI polyamide conjugate alkylated the target site. However, the detailed information of the recognition sequence was still not clear in this experiment.

Preparation of a Five-Well DNA Frame and a dsDNA-Attached Frame. For the precise analysis, we designed and prepared a novel observation scaffold. The five-well DNA frame (5WF) was prepared by mixing M13mp18 single-stranded DNA and the designed staple strands in Tris-HCl buffer (pH 7.6) containing Mg^{2+} and EDTA.[9] To promote annealing of the DNA, the temperature of the solution was decreased from 85 to 15 °C at a rate of −1.0 °C/min. The 5WF scaffold was deposited on the mica surface and imaged in buffer solution using AFM (Figure 4A). The 5WF scaffold was formed as designed. The size of the 5WF in the AFM image was ∼110 nm × ∼90 nm, which is similar to the size expected from the original design. The five cavities were clearly evident and were located in the predesigned places on the planar structure. The size of the cavities was ∼20 nm × ∼15 nm. For the introduction of five different sequences of 64-mer dsDNAs to the corresponding cavities, 5′ overhangs (16-mer single-stranded DNA) complementary to both edges of the cavity were used (Figure 4B and detailed sequences in Supporting Information). To examine the selective incorporation of the dsDNA to the corresponding well, dsDNA-III was conjugated to the 5WF scaffold by annealing from 60 to 15 °C at a rate of −0.5 °C/min. Selective attachment to the well-III and no incorporation of dsDNA to other wells were observed by AFM (Figure S2). Then, annealing of the five 64-mer dsDNAs to the 5WF scaffold was examined using the same conditions. The resulting products were analyzed, and ∼80% of the 5WF scaffold was filled with five dsDNAs (Figure 4C). The method is also adequate for the preparation of nanostructures having the multiple dsDNA substrates for single-molecule analysis.

Single-Molecule Alkylation to Specific Sequences in the Five-Well DNA Frame. The selective alkylation to dsDNAs in the 5WF scaffold by conjugate **1** was observed using AFM. Five 64-mer dsDNAs were introduced to the individual

dx.doi.org/10.1021/ja209023u | *J. Am. Chem. Soc.* 2012, 134, 4654−4660

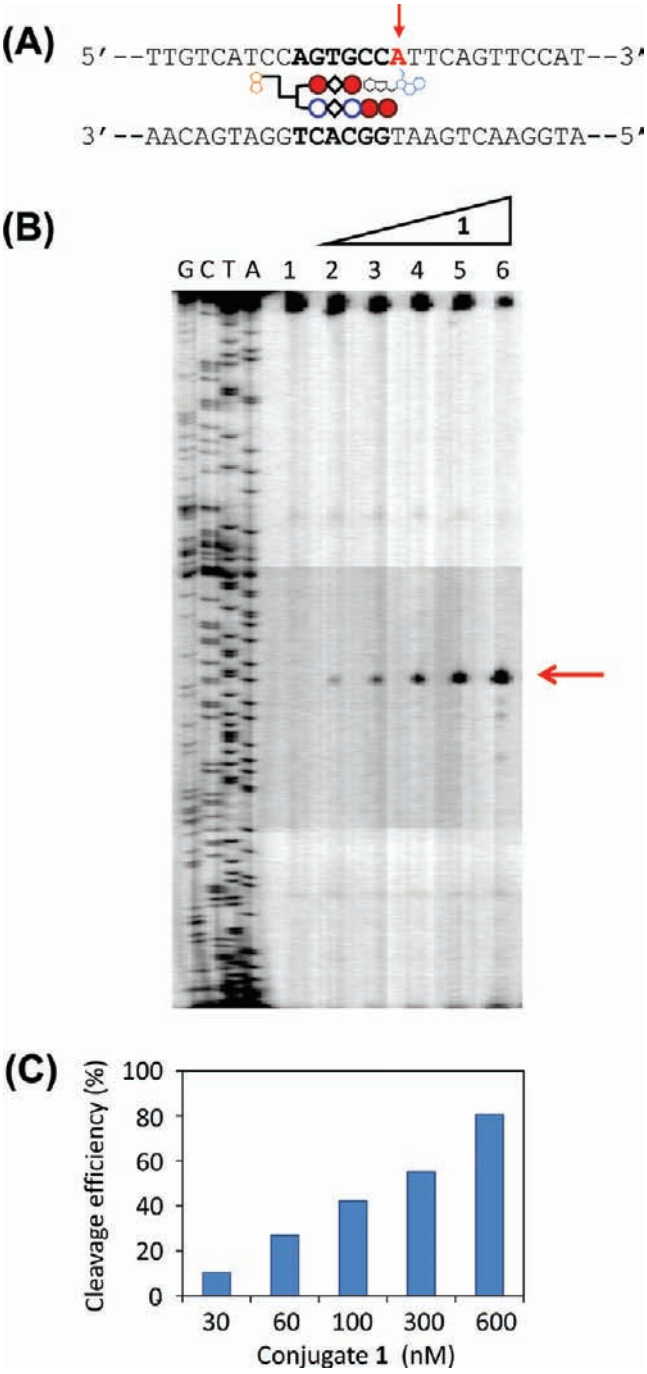


Figure 2. Analysis of the sequence-selectivity of PI polyamide conjugate **1** by sequencing polyacrylamide gel electrophoresis (PAGE). (A) Binding location of PI polyamide conjugate **1**, the recognition sequence, and the alkylation site in the target DNA. (B) Sequencing PAGE for identification of an alkylation site by conjugate **1**. Thermally induced DNA strand cleavage of the DNA fragment (208 bp) containing target sequence 5′-AGTGCCA-3′ after incubation with conjugate **1** at 23 °C for 24 h. The 5′-end of the DNA fragment was labeled with Texas Red. Lane 1, negative control in the absence of conjugate **1**; lanes 2−6, incubation with 30, 60, 100, 300, 600 nM of conjugate **1**, respectively. Alkylation site is shown with a red arrow. (C) Quantification result of the cleavage by alkylation with various concentrations of conjugate **1**.

wells of the 5WF scaffold (Figure 1C), where four dsDNAs (I−IV) contained the sequences AGTXCCA (X = A, T, G, C) with fully matched [X = G (III)] and one-base mismatched [X = A (I), T (II), C (IV)] nucleotides, and one dsDNA (V) had the sequence AGTGCCT, which lacked an alkylating site. The 5WF filled with dsDNAs was incubated with conjugate **1** at 25 °C for 24 h, and then the sample was placed on the mica surface in the buffer. To remove excess conjugate **1**, the mica plate was washed with the buffer, and then a solution of streptavidin was applied onto the mica plate. The binding of streptavidin to the 5WF was observed using AFM (Figure 5). Streptavidin preferentially bound to the well-III, where the full-match sequence for the recognition was introduced (Figure S2). Streptavidin was detected on 4.8% of dsDNA-III and on 0.7% of the dsDNA-IV (Figure 6), and no streptavidin was present on dsDNA-I and dsDNA-II. Therefore, the affinity of the Im−Py pair for the G−C pair was directly examined by replacing the guanine moiety in the center of the recognition sequence to adenine (dsDNA-I) or thymine (dsDNA-II), and the selectivity for the target sequence was 88%. These results demonstrate that the PI polyamide discriminates one-mismatched nucleotide at the single-molecule level.

We also examined the requirement for alkylation by removing the alkylation site. The sequence of dsDNA-V contained fully matching recognition sequence, but this lacked the adenine for alkylation. When binding of the PI polyamide was compared using dsDNA-III and dsDNA-V, the requirement of alkylation could be revealed. As shown in the result, binding of the PI polyamide to dsDNA-V was not detected, indicating that alkylation anchored the PI polyamide to the target dsDNA.

Some streptavidin labeling was found at the edges of the frame. Because the edges face the solution, some nonspecific binding of PI polyamide can occur. This was confirmed by introduction of the alkylation products of dsDNAs I−V with conjugate **1** to the 5WF using the same assembly conditions. Streptavidin labeling was only detected on the fully matched dsDNA-III (5.8% of total 5WF). We observed the good sequence-selectivity of PI polyamide; however, the yield was still low. The lower yield of streptavidin labeling compared with the alkylation analyzed by PAGE may be attributed to the partial depurination of the PI polyamide−alkylating agent−adenine adduct under the sample preparation. The depurination and subsequent strand cleavage preferentially occur at higher temperature; however, this could occur at room temperature depending on the incubation time.[17] The length and position of the biotin-linker moiety may affect the reactivity and streptavidin labeling efficiency. Because the biotin-PEG linker is conjugated to the hairpin region of the PI polyamide, binding of the PI polyamide to the target sequence may be less than that of a biotin-polyamide conjugated to the terminal region.[15]

In the previous report, the double-crossover DNA tile (DX) system was used for streptavidin-labeling of DNA nanostructures using PI polyamide. PI polyamides bound to the target sequences were clearly observed and these can thus be used for addressing single-molecules.[12] When using the DX system, it is difficult to analyze the sequence-selectivity of PI polyamide conjugates because the DX tile system dose not provide a wide variety of recognition sequences in one structure, which is important for screening the multiple recognition sequences at the same time.

■ CONCLUSION

We have demonstrated a novel method for monitoring single-molecule alkylation of a target dsDNA using a synthetic PI

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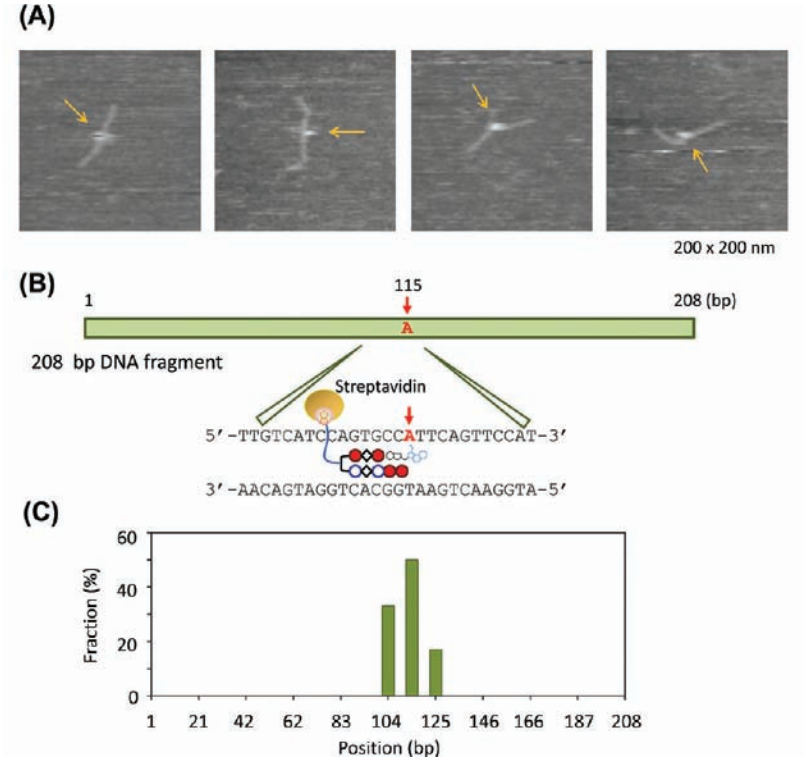

Figure 3. AFM observation for alkylation of the 208 bp DNA fragment with conjugate **1**. (A) AFM images of streptavidin-labeling after alkylation with conjugate **1**. (B) Schematic representation of the position of the alkylation site in the DNA fragment and the target sequence. (C) Distribution of the position of streptavidin in the 208 bp DNA fragment.

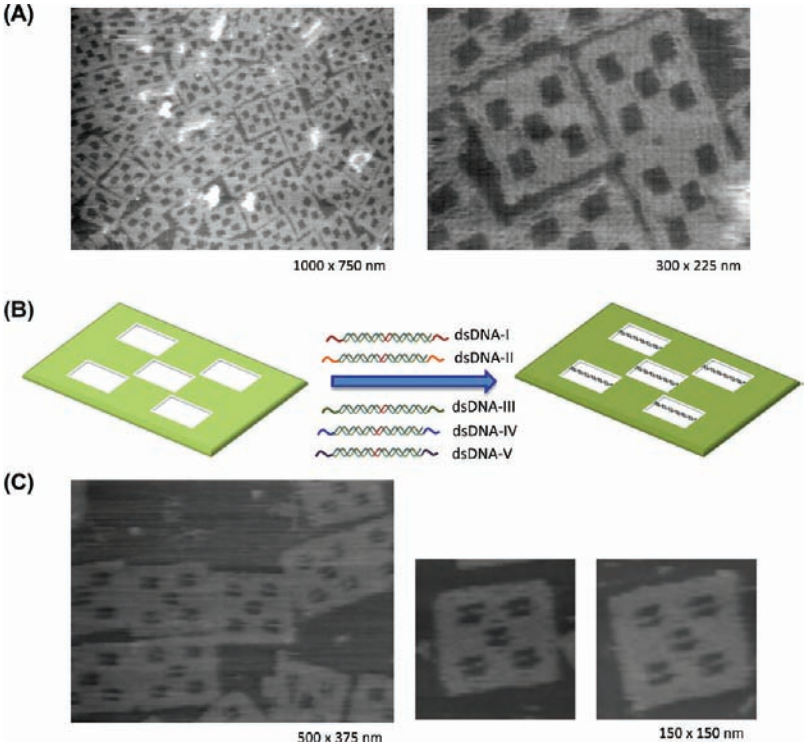

Figure 4. Assembly of five-well DNA frame and incorporation of five dsDNAs. (A) AFM images of 5WF scaffold. (B) Incorporation of five different 64-mer dsDNAs into the 5WF scaffold by self-assembly. (C) AFM images of five-dsDNA-attached 5WF.

polyamide in a purpose-designed DNA origami scaffold. The 5WF scaffold enabled incorporation of five different dsDNAs for analysis of the sequence-selective recognition and alkylation of the PI polyamide. We observed the binding of the PI polyamide to the dsDNAs incorporated in the 5WF scaffold. The PI polyamide retained its recognition and alkylation ability at the single-molecule level and showed 88% selectivity for the full-matched sequence over other mismatched sequences. We

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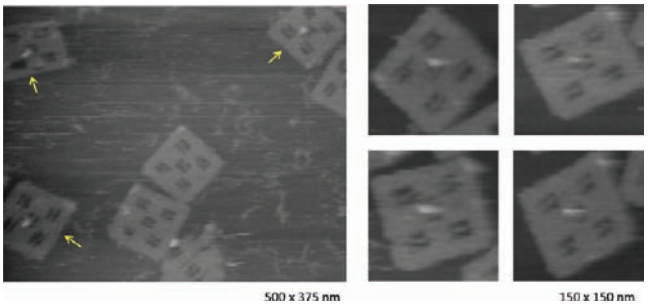

Figure 5. Sequence-selective single-molecule alkylation with conjugate **1**. AFM images for the alkylation in the 5WF carrying five dsDNAs. Alkylation of dsDNA in the 5WF was carried out with conjugate **1** at 23 °C for 24 h, and then the 5WF was labeled with streptavidin. The four images on the right are magnified images, where streptavidin is attached to the dsDNA-III in the well-III.

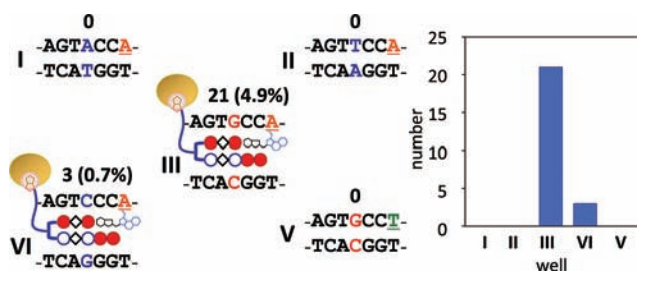

Figure 6. Results of sequence-selective alkylation with conjugate **1**; the number of streptavidin observed in the specific wells in the 5WF (426 5WFs were counted). The graph shows the number of the wells where the dsDNA was labeled with streptavidin.

revealed that the selective alkylation using PI polyamide conjugates can inhibit the transcription *in vitro* and *in vivo*.[18] The combination of selective alkylation and single-molecule observation can be used for the analysis of the effect of alkylation on the reactivity and binding activity of the DNA binding enzymes and proteins. The designed DNA origami scaffold can accept the various unnatural nucleotide substrates including nucleotide analogues, damaged bases, and mismatched-base pairs, and these target substrates are easily installed by just replacing the dsDNAs to be incorporated.[9] In addition, the physical properties of dsDNA such as tension, rotation, and topology are controlled in the nanoscale cavities that the DNA origami scaffold provides.[9] Therefore, the single-molecule observation system provides novel information on relationships between the biochemical and physical properties of substrate dsDNA and the molecules of interest including synthetic ligands and enzymes.

MATERIALS AND METHODS

Materials. Reagents and solvents for the synthesis were purchased from standard suppliers and used without further purification. BioTOF II (Bruker Daltonics) mass spectrometer was used for analyses by electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS). Polyacrylamide gel electrophoresis was performed using a HITACHI SQ5500-E DNA sequencer. The gel-filtration column and the Sephacryl S-300 were purchased from BioRad Laboratories (Hercules, CA) and GE Healthcare (Buckinghamshire, U.K.), respectively.

Synthesis of AcImImPy-β-Py-(*R*)^{H2N}γ-Im-β-Im-CO_2H (2). Py–Im polyamide was synthesized according to the previously reported methods.[19] AcImImPy-β-Py-(*R*)^{HNBoc}γ-Im-β-Im-CO_2-CLEAR-Acid resin was treated with 1 mL of TFA/water/triisopropylsilane (95/2.5/2.5; v/v/v) at room temperature (rt) for 1 h to cleave from the solid-phase support and remove the Boc group.[20] The solution was evaporated, the residue was dissolved in CH_2Cl_2/MeOH (1/1), and the target product **2** was obtained by recrystallization from diethylether as white powder (19.6 mg, 18.2 μmol, 53%). Compound **2** was used for synthesis of conjugate **3** without further purification. ESI-TOFMS *m*/*z*: calcd for $C_{44}H_{54}N_{20}O_{11}$ $[M + H]^+$, 1039.44; found, 1039.42.

Synthesis of AcImImPy-β-Py-(*R*)$^{HN\text{-}PEG12\text{-}Biotin}$γ-Im-β-Im-$CO_2H$ (3). NHS-PEG_{12}-Biotin (1.7 mg, Thermo) was dissolved in DMF (150 μL), to which *i*Pr_2NEt (1.9 μL, 10.8 mmol, 6.0 equiv) and compound **2** (2.0 mg, 1.8 μmol, 1.0 equiv) were added, and stirred at rt for 3 h. After recrystallization from diethylether, compound **3** was obtained as yellow oil (3.5 mg, 1.9 μmol, quant.). ESI-TOFMS *m*/*z*: calcd for $C_{81}H_{121}N_{23}O_{26}S$ $[M + 2H]^{2+}$, 932.94; found, 932.99. Compound **3** was used for the synthesis of conjugate **1** without further purification.

Synthesis of AcImImPy-β-Py-(*R*)$^{NH\text{-}PEG12\text{-}Biotin}$γ-Im-β-Im-Indole-*seco*-CBI (1). To a solution of compound **3** (3.5 mg, 1.9 μmol) in 50 μL of DMF was added *i*Pr_2NEt (2.0 μL, 11.3 μmol, 6.0 equiv) and PyBOP (2.0 mg, 3.8 μmol, 2.0 equiv), and stirred at rt for 3 h. Indole-*seco*-CBI was added to the mixture, and the reaction mixture was stirred at rt for 24 h. After washing with diethylether, the resultant was purified using a reversed-phase HPLC with a Chemcobond 5-ODS-H column (10 × 150 mm) (H_2O with 0.1% AcOH containing 30–75% CH_3CN over linear gradient for 30 min at a flow rate of 3 mL/min). The peak was collected and lyophilized. Conjugate **1** was obtained (0.6 mg, 0.27 μmol, 14%) as a white powder. ESI-TOFMS *m*/*z*: calcd for $C_{103}H_{137}ClN_{26}O_{27}S$ $[M + 2H]^{2+}$, 1119.49; found, 1119.50.

Polyacrylamide Gel Electrophoresis for Analysis of Selective Alkylation with PI Polyamide Conjugate. The 5′-Texas Red-modified 208 bp DNA fragments (6 nM) were alkylated by the biotinylated Py–Im polyamide-*seco*-CBI conjugate **1** in 10 μL of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 23 °C for 24 h. The reaction was quenched by adding calf thymus DNA (10 mg/mL, 1 μL), and the mixture was heated at 95 °C for 5 min. The solution was concentrated by vacuum centrifugation. The pellet was dissolved in 6 μL of loading dye (formamide with New fuhsin) and heated at 95 °C for 25 min, and then immediately cooled to 0 °C. A 1.2 μL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a DNA sequencer. Electrophoresis was conducted under 1.5 kV, ca. 25 mA, and 40 °C. The gel image was quantified by Fujifilm multi gause software.

Preparation of the Five-Well Frame and the dsDNA-Attached Five-Well Frame. The DNA five-well frame was designed using caDNAno software (Supporting Information).[6b] The DNA five-well frame was assembled in 20 μL of solution containing 10 nM M13mp18 single-stranded DNA (New England Biolabs), 50 nM staple strands (5 equiv), 20 mM Tris buffer (pH 7.6), 1 mM EDTA, and 10 mM $MgCl_2$, following the previous study.[9] The mixture was annealed by reducing the temperature from 85 to 15 °C at a rate of −1.0 °C/min. The dsDNAs to be incorporated were prepared by annealing of the corresponding DNA strands, and dsDNAs (2 equiv excess) were incorporated into the five-well DNA frame by heating at 60 °C and then cooling to 15 °C at a rate of −0.5 °C/min using a thermal cycler. The samples were purified using Sephacryl S-300 gel-filtration column.

Alkylation of DNA Fragment and dsDNAs Attached to the Five-Well DNA Frame. For the alkylation of DNA fragment (208 bp), the target dsDNA (6 nM) was incubated with PI polyamide conjugate **1** (100 nM) in 10 μL of a buffer solution containing 20 mM Tris buffer (pH 7.6), 1 mM EDTA, 10 mM $MgCl_2$, and 10% DMF at 25 °C for 24 h. In the case of the substrate in the 5WF, target dsDNAs in the 5WF (10 nM) were alkylated by PI polyamide conjugate **1** (100 nM) in 10 μL of the same buffer at 25 °C for 24 h.

AFM Imaging. AFM images were obtained using a fast-scanning AFM system (Nano Live Vision, RIBM Co. Ltd., Tsukuba, Japan) with a silicon nitride cantilever (resonant frequency =n1.0–2.0 MHz, spring constant = 0.1–0.3 N/m, EBD Tip radius <15 nm, Olympus BL-AC10EGS-A2). The sample (2 μL) was adsorbed onto a freshly cleaved mica plate (ϕ 1.5 mm, RIBM Co. Ltd., Tsukuba, Japan) for 5

dx.doi.org/10.1021/ja209023u | *J. Am. Chem. Soc.* 2012, 134, 4654–4660

min at rt and then washed three times using the same buffer solution. Solution of streptavidin was applied onto the mica plate, followed by washing three times using the same buffer solution. Scanning was performed by tapping mode in the same buffer solution.

AFM image analysis for the alkylated 208 bp DNA fragment was performed using ImageJ software for measurement of the position of streptavidin bound to the DNA fragment.

■ ASSOCIATED CONTENT

Supporting Information

Characterization of PI polyamide conjugates and design of five-well frame and staple strand sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Core Research for Evolutional Science and Technology (CREST) of JST and a Grant-in-Aid for Science research from MEXT, Japan. Financial supports from Sumitomo Foundation and Iketani Science and Technology Foundation to M.E. are also acknowledged.

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