DNA Recognition

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Conjugation of Peptide Nucleic Acid with a Pyrrole/Imidazole Polyamide to Specifically Recognize and Cleave DNA**

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Recognition and incision of sequences within double-stranded DNA (dsDNA) are widely used in gene manipulation.[1] Chemistry-based approaches have exploited base-pairing for sequence-specific recognition of DNA.[2–12] Several research groups, including our own, have reported that a pseudocomplementary peptide nucleic acid (pcPNA) effectively recognizes complementary A/T-rich sequences by invading dsDNA.[13–15] In addition, we have demonstrated that a single PNA forms a PNA/DNA hybrid G-quadruplex with the G-rich region in dsDNA.[16] Recently, we also successfully used a pcPNA to determine the length of telomere DNA by breaking the target sequence.[17] Although these modified PNAs can target DNA sequences using a double-duplex invasion strategy, successful invasion appears to require relatively low salt concentrations.[18–21] At physiological ionic strengths, little to no invasion has been reported.[22] Furthermore, PNAs only recognize shorter sequences, and thus reduce their sequence specificity and limits their utility. For example, a PNA-Hoechst conjugate was shown to target an A/T region, in terms of targetable sequences for the Hoechst moiety (Hoechst shows with preference for A/T rich sequences only), and sequence-wide selectivity is far from optimal.[23] Therefore, an effective chemical method that overcomes these limitations and efficiently recognizes DNA sequences is desired.

Herein we present a novel approach for targeting double-stranded DNA. The method is successfully applied to sequence-specific DNA invasion and cleavage of the target sequence. As shown in Figure 1, dsDNA is recognized by a combination of double-duplex invasion using pcPNA (green lines) and minor groove binding using a pyrrole/imidazole (Py-Im) polyamide (red and blue balls). The Py-Im polyamide is known to bind to the minor groove of DNA through multiple hydrogen bonds.[24–26] By using the pcPNA1-polyamide conjugate strategy, we accomplished 1) DNA binding under high salt conditions and 2) specific cleavage of long dsDNA.

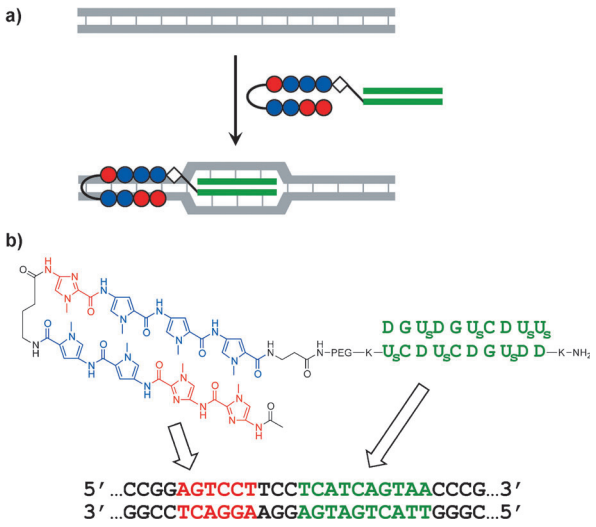

Figure 1. a) Recognition of double-stranded DNA by combining the pcPNA (decamer) invasion and Py-Im polyamide binding (recognizing 6 bp DNA). b) Structure of the pcPNA/hairpin polyamide conjugate. DNA sequences recognized by each portion are presented. PEG = polyethyleneglycol.

The conjugate **1** was prepared using continuous solid-phase synthesis, as shown in the Supporting Information (Figure S1). DNA binding activity was assessed by a gel-shift assay using a 130 bp dsDNA (DNA1). The pcPNA1 portion in **1** is complementary to one strand of DNA1 (Figure 2). The double-duplex invasion complex is formed by the addition of pcPNA2, which is complementary to the other strand of DNA1. In contrast, the polyamide portion of **1** binds to the 5′-AGTCCT-3′/3′-TCAGGA-5′ site of DNA1. As shown in lane 3 of the gel, a new low-mobility band was clearly and

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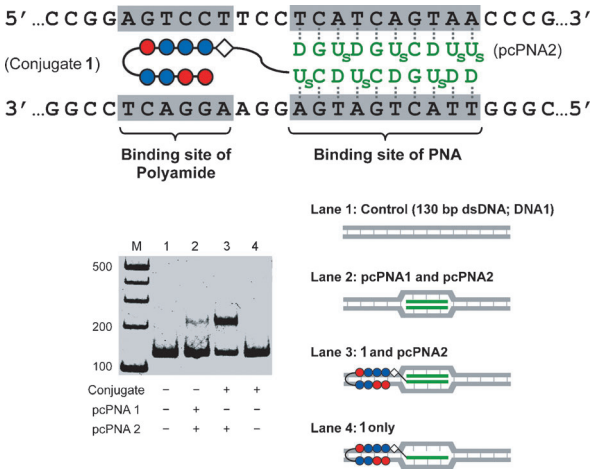

Figure 2. Gel-shift assay for binding of the combination of **1** and pcPNA2 to dsDNA. The pcPNA binding site (5′-TCATCAGTAA-3′) and the polyamide binding site (5′-AGTCCT-3′) in the DNA1 are shown. Lane 1: DNA1 only; lane 2: DNA1 with pcPNA1/pcPNA2; lane 3: DNA1 with **1**/pcPNA2; lane 4: **1** only; M: 100 bp ladder. Invasion conditions: [DNA1] = 10 nM, [**1**] = 100 nM, [pcPNA1 or 2] = 100 nM, [HEPES] = 5 mM, and [NaCl] = 20 mM at pH 7.0 and 50 °C for 24 h.

almost completely formed when **1** was combined with pcPNA2 (Figure 2). The binding efficiency of pcPNA1 and pcPNA2, without **1**, was intrinsically weakened (lane 2). In the absence of pcPNA2, no new band was formed (lane 4). Therefore, we infer that the invasion complex formed by the pcPNAs and the binding of the minor groove and polyamide successfully function in a cooperative manner.

Subsequently, we investigated the DNA-binding ability of the **1** under high salt conditions. When **1** and pcPNA2 were combined in 50 mM KCl aqueous solution, 69 % of the DNA1 was bound (lane 5; Figure 3a). As the KCl concentration increased to 100 and subsequently to 150 mM, the binding was still markedly observed (lanes 6 and 7). In contrast, the binding of the pcPNA1/pcPNA2 combination (without **1**) was weakened and considerably more sensitive to increasing salt concentration. In the absence of **1**, only 29 % of the DNA bound to the invasion complex at 50 mM KCl (lane 2). As the salt concentration increased (100 mM in lane 3 and 150 mM in lane 4), invasion reduced to the point of being unobservable. Under these conditions, the decameric pcPNAs are too short to efficiently form double-duplex invasion complexes without assistance from the Py-Im polyamide. The superior DNA-binding capacity of the **1**/pcPNA2 combination compared to that of pcPNA1/pcPNA2 at increased salt concentrations became more evident when KCl was replaced with $MgCl_2$ (Figure 3b). In the presence of 1–2 mM Mg^{2+}, most of the DNA1 was bound to the **1**/pcPNA2 combination (lanes 6 and 7). In contrast, approximately 30 % and 60 % of the pcPNA/pcPNA2 combination dissociated at $MgCl_2$ concentrations of 1 and 2 mM, respectively (lanes 3 and 4).

To further evaluate the efficacy of the **1**, we performed a binding experiment under physiological salt conditions. The saline conditions of living cells were mimicked by adding NaCl (12 mM), KCl (139 mM), and $MgCl_2$ (0.8 mM; Figure 3c). Gel-shift analysis revealed that, even under these conditions, a notable amount of DNA1 was bound to **1**/pcPNA2 (lane 3). The pcPNA1/pcPNA2 combination failed to bind to the dsDNA (lane 2). These results suggest that successful DNA invasion requires **1**, thus indicating the important role of minor-groove binding by a polyamide in the PNA invasion of dsDNA.

Sequence-specific binding of **1** was evaluated using a mismatch DNA. In the sequence-specificity test, the C/G pair in DNA1 was changed to an A/T pair in DNA3 (shown in green in Figure 4a), and a single-base-pair mismatch was introduced between the DNA and the polyamide binding site. The **1**/pcPNA2 combination almost completely bound to fully-matched DNA (DNA1; lane 2 in Figure 4b). In contrast, as shown in lane 3, the binding between **1**/pcPNA2 and the mutated DNA was greatly weakened, with only 19 % of the DNA bound. According to the binding efficiency (Figure 4c), **1** sequence-specifically recognized the dsDNA.

Subsequently, we examined the influence of the number of bases between the pcPNA binding site and the polyamide binding site on DNA binding. The number of bases was systematically changed, as shown in Figure S3. The following order of binding efficiency was obtained: CC in DNA2 < TCC in DNA1 > GTTCC in DNA4. The optimal distance between the pcPNA binding site and the polyamide binding site was three base pairs.

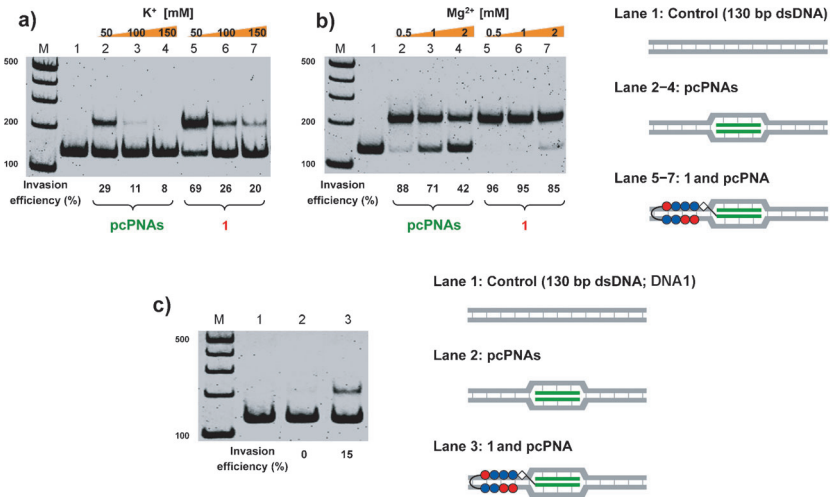

Figure 3. a,b) Binding of DNA1 (130 bp) with **1**/pcPNA2 at higher salt conditions. Lane 1: DNA1 only; lanes 2–4: DNA1 with pcPNA1/pcPNA2 (without the use of the polyamide); lanes 5–7: DNA1 with **1**/pcPNA2; M: 100 bp ladder. Invasion conditions: [DNA1] = 10 nM, [**1**] = [pcPNA1] = [pcPNA2] = 100 nM, [HEPES] = 5 mM at pH 7.0 and 37 °C for 20 h. c) Gel-shift assay for DNA binding under physiological salt conditions ([NaCl] = 12 mM, [KCl] = 139 mM and [$MgCl_2$] = 0.8 mM). Lane 1: DNA1 only; lane 2: DNA1 with pcPNA1/pcPNA2 (200 nM each); lane 3: DNA1 with **1**/pcPNA2 (200 nM each); M: 100 bp ladder. Invasion conditions: [DNA1] = 10 nM, [HEPES] = 5 mM at pH 7.0 and 37 °C for 20 h.

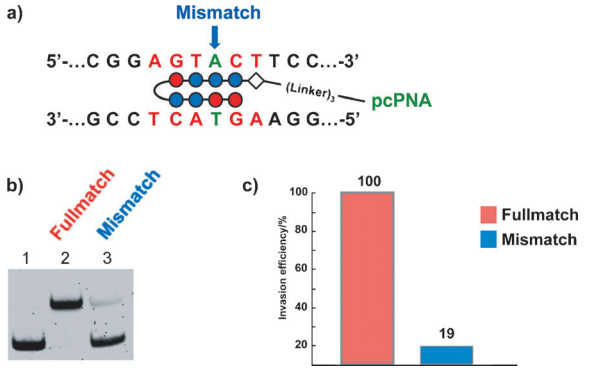

Figure 4. Mismatch recognition by the polyamide portion of **1**. a) In DNA3, the C/G base pair in the polyamide binding site of DNA1 was changed to A/T (green). b) Gel-shift assay. Lane 1: DNA1 only; lane 2: DNA1 with **1**/pcPNA2; lane 3, DNA3 (having a single-base pair mismatch) with **1**/pcPNA2. c) Binding efficiency of **1**/pcPNA2 to DNA1 (red) and DNA3 (blue). Invasion conditions: [DNA1] = [DNA3] = 10 nM, [**1**] = [pcPNA2] = 100 nM, [HEPES] = 5 mM, and [$MgCl_2$] = 1 mM at pH 7.0 and 37 °C for 20 h.

We further evaluated the interaction between the Py-Im portion and dsDNA using the surface plasmon resonance (SPR) technique with biotinylated hairpin DNA. Dissociation equilibrium constants (K_D) of Py–Im polyamide (**2**) with full-match and mismatch DNAs were obtained by fitting the resulting sensorgrams to a theoretical model (see Figure S4).[27,28] The K_D value for **2** with full-match DNA was determined as 3.71×10^{-7} M, which corresponds to a significantly higher binding affinity compared with the **2**/mismatch DNA system ($K_D = 1.22 \times 10^{-5}$ M). **2** binds to full-match DNA with fast association kinetics and a k_a value of $7.8 \times 10^3\ M^{-1} s^{-1}$, relative to mismatch DNA, with a k_a value of $8.6 \times 10^2\ M^{-1} s^{-1}$.

Having confirmed the efficient binding of **1** to dsDNA, we were encouraged to attempt sequence-specific double-strand cleavage in a long DNA strand. The target DNA sequence was cleaved by recruiting the catalytic Ce^{IV}/EDTA species to the target site.[29] To achieve this, L-phosphoserine, a monophosphate group, was attached to the N-terminus of pcPNA2 (designated pcPNA3 in Figure 5). Because phosphonate-type ligands exhibit high affinity toward lanthanide ions, PNAs bearing this terminal group efficiently recruit the catalytic Ce^{IV}/EDTA species to the target site, thus resulting in efficient cleavage. We examined site-selective scission of a long dsDNA (DNA5; 4366 bp). The 5′-TCATCAGTAA-3′/3′-AGTAGTCATT-5′ site of DNA5 is the target for the double-duplex invasion of **1**/pcPNA3, while the 5′-AGTCCT-3′/3′-TCAGGA-5′ site is the polyamide binding site. If scission occurs at the binding site of the **1**/pcPNA3 combination, two fragments of approximately 1840 and 2530 bp should be formed. Gel electrophoresis (lane 3 of Figure 5) revealed two fragments of the expected sizes. However, in the absence of the **1**/pcPNA3 combination these bands were absent (lane 2). These results suggest that the cooperation between pcPNA and the Py-Im polyamide plays a key role in the site-selective scission of dsDNA.

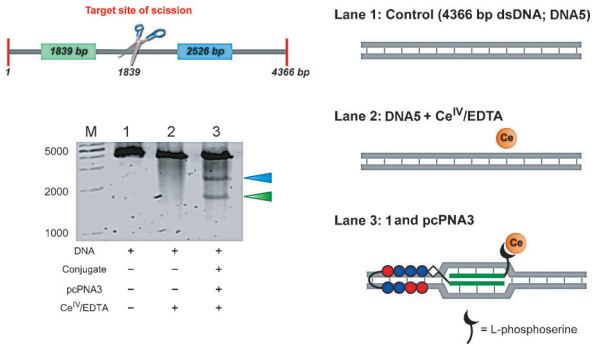

Figure 5. Site-selective hydrolysis of dsDNA (4366 bp; DNA5) with and without **1**/pcPNA3. Lane 1: control [in the absence of both Ce^{IV}/EDTA and **1**]; lane 2: with Ce^{IV}/EDTA only; lane 3: with Ce^{IV}/EDTA in the presence of **1**/pcPNA3; M: 1000 bp ladder. Reaction conditions: [DNA5] = 4 nM, [**1**] = [pcPNA3] = 200 nM, [Ce^{IV}/EDTA] = 200 μM, [NaCl] = 100 mM, and [HEPES] = 5 mM at pH 7.0 and 50 °C for 16 h.

In summary, the present study provides a simple approach to resolve the technical difficulties in targeting dsDNA using PNAs in the presence of ion concentrations present under physiological conditions. In fact, the problem of strand invasion under physiological conditions remains a major challenge in dsDNA recognition. Moreover, we have shown that the method was successfully applied to sequence-specific DNA scission for a longer target sequence. The chemical strategy allows strand invasion to take place in a sequence-specific manner under high salt conditions and may serve as a new technology for gene manipulation.

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