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Site-selective DNA hydrolysis induced by a metal-free peptide nucleic acid–cyclen conjugate†

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A metal-free artificial restriction DNA cutter which is composed of cyclen and classical peptide nucleic acid (PNA) was synthesized. Analysis of DNA cleavage products indicates the site-selective hydrolysis.

DNA is one of the most important biomacromolecules in all living organisms, which contains the genetic information. Human diseases such as cystic fibrosis, Alzheimer's, sickle cell anemia, and certain cancers are associated with mutations in the sequence of particular genes.[1] However, the half-life of DNA by spontaneous hydrolysis under physiological conditions is estimated as thousands to billions of years which is primarily due to the repulsion between the negatively charged backbone and potential nucleophiles.[2] Therefore, studies of DNA cleaving agents are of great interest and importance for their potential biotechnology applications. In this field, site-selectivity is an especially challenging goal even though it is common in nature. To date, it has been achieved mainly by three systems: (1) restriction enzymes; (2) DNAzymes; (3) chemistry-based artificial nucleases. Although restriction enzymes can recognize DNA sequences and are commercially available, they have significant drawbacks such as short recognition sequences which cannot ensure high specificity of cleaving large DNA molecules. DNAzymes refer to single-stranded DNA with DNA/RNA cleaving ability which is obtained through *in vitro* selection from random DNA libraries.[3] All the DNA-cleaving DNAzymes obtained up to now need specific metal ions as cofactors for sequence-specific cleavage.

Chemistry-based artificial nucleases are basically composed of two key components, a sequence-recognizing moiety and a DNA-cutting agent.[4g] Recently, peptide nucleic acid (PNA) based artificial nucleases have achieved remarkable success.[4–7] Krämer and co-workers described a family of PNA–metal chelating conjugates whose Zr(IV) complexes could site-selectively hydrolyze single-stranded DNA.[6] Very recently, double-stranded DNA cleaving has been achieved by artificial restriction DNA cutters (ARCUTs), which combine Ce(IV)/EDTA with two pseudo-complementary peptide nucleic acid (pcPNA) additives, in Komiyama's laboratory.[4] However, in the previous work, DNA cleavage was typically observed under non-physiological conditions (*e.g.*, 22 °C or 50 °C). And meanwhile, most efforts were focused on the metal complexes, which may cause metal dissociation and uncontrolled redox chemistry.[8] In the absence of metal ions, cleavage is considered to be much safer for applications. Herein, we present a study on the water soluble 1,4,7,10-tetraazacyclododecane (cyclen) tethered PNA tetramer conjugate possessing features that are required for metal-free site-selective hydrolysis of DNA under physiological conditions (Fig. 1).

The target conjugate of the PNA tetramer and cyclen was prepared by a standard liquid-phase peptide synthesis protocol as previously described[9] and has been characterized by MALDI-TOF mass spectrometry and HPLC. It was found to be water soluble and quite stable. A detailed synthesis route is given in the ESI† (S-1).

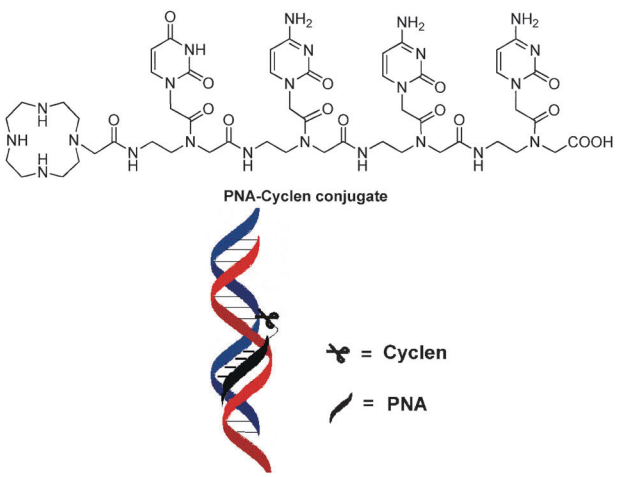

Fig. 1 Structure and schematic view of the PNA–cyclen conjugate used for site-selective cleavage of the DNA.

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DNA binding ability is a critical step for subsequent cleavage, and DNA melting experiment is a straightforward way to investigate the relative binding ability of PNA–cyclen that interacts with calf thymus DNA (CT-DNA).[10] As shown in Fig. S1 (ESI†), the duplex CT-DNA alone melted at 76.4 °C, whereas the duplex gains a stabilization of 9 °C in the presence of the PNA–cyclen conjugate. As cyclen itself did not stabilize CT-DNA apparently,[10] the absorbance changes observed at 260 nm clearly revealed the increased thermal stability of PNA–DNA duplexes relative to the corresponding DNA–DNA duplexes. On the other hand, the binding constant was further determined by evaluating the fluorescence emission intensity of the ethidium bromide (EB)–DNA system (Fig. S2†). Addition of the conjugate to the DNA-bound EB solution caused an appreciable reduction in the emission intensity. The quenching constant K_{sv} given by the slope of the plot was estimated as 0.39×10^3 M^{-1}. Upon comparison, cyclen itself showed almost no change in the fluorescence intensity (Fig. S2b†). Moreover, the apparent binding constant K_{app} was also calculated as 4.8×10^3 M^{-1} for the PNA–cyclen conjugate. This binding constant implies the stability of PNA–DNA duplex.

The DNA cleaving ability was assayed by using supercoiled pUC19 plasmid DNA as a substrate in Tris-HCl (50 mM, pH 7.4) in the absence of any external agents or metal ions and determined by agarose gel electrophoresis. Under these conditions, after 24 h of incubation at 37 °C, a 0.57 mM PNA–cyclen conjugate completely cleaved supercoiled DNA to open circular form (Fig. 2, Lane 6). In order to avoid any effect due to residual metal contamination in the sample, a potent metal chelating agent (6 mM EDTA) was introduced into the system. No evidence indicated that inhibition of DNA cleavage occurred (Lane 5). However, a similar assay using cyclen alone did not show any apparent cleavage (Lane 4). For further demonstrating that the DNA cleavage was because of the metal-free cyclen motif domain, supplementary experiments were carried out by using a 3Boc-protected cyclen tethered PNA tetramer as cleaving agent (Fig. S4, ESI†). Almost no cleavage activity was observed. Results demonstrate that the DNA cleavage is initiated by the cyclen derivative when PNA was introduced.

Further investigations of the cleaving ability have been carried out through a series of optimization experiments including concentration, ionic strength, pH value and reaction time. The percentages of supercoiled DNA decreased with the increasing compound concentration (Fig. S5, ESI†). Higher concentration would condense DNA due to its strong binding affinity and resulted in partial block of the plasmid in the electrophoresis migration (data not shown). The lack of electrostatic repulsion between DNA and neutral PNA leaves the binding affinity independent of salt concentration.[11] Therefore, increasing the ionic strength of reaction buffer would not influence the cleavage activity (Fig. S6†). The PNA–cyclen conjugate catalyzed cleavage in pH range 6.0–9.0 was also studied (Fig. S7†), and the results showed that the amount of cleaved DNA increased with the pH of the solution, which could be the result of more active nucleophiles formation by the deprotonation of water.[12,13] The process of DNA cleavage perhaps was related to H_2O molecules activated by the cyclen motif.[14] Furthermore, time-course study of DNA cleavage was carried out. Data shown in Fig. S8 (ESI†) were fit to the integrated equation of pseudo first order reaction to obtain the rate of DNA cleavage, $k_{obs} = 0.185 \pm 0.014$ h^{-1}. It is about 10^7 times higher than that of DNA natural degradation.[2]

To verify the mechanism of DNA cleavage promoted by the PNA–cyclen conjugate, typical reactive oxygen species (ROS) scavengers[13] including hydroxyl radical scavengers (DMSO and *t*-BuOH), singlet oxygen scavenger (NaN_3) and superoxide scavenger (KI) were added to the system. As shown in Fig. 3 and Fig. S9 (ESI†), no evident inhibition effect on the DNA cleavage was observed in the presence of any scavenger. The results ruled out the involvement of cleavage by ROS species, indicating that the DNA cleavage may occur *via* a hydrolytic pathway. In addition, the ligation experiment of cleaved pUC 19 DNA further provided the evidence for a hydrolytic mechanism. After the cleaved DNA fractions were recycled by cutting off the gel and then incubated with T4 ligase for 12 h at 25 °C, most of the DNA fractions were re-ligated successfully (Fig. 3b, lane 4). The results clearly suggested that the PNA–cyclen conjugate did hydrolyze DNA.

The denaturing polyacrylamide gel electrophoresis (PAGE) experiment was performed with the aim of investigating the sequence selectivity. A 24-nt hairpin oligonucleotide incorporating only one potential binding and cleavage site chosen as

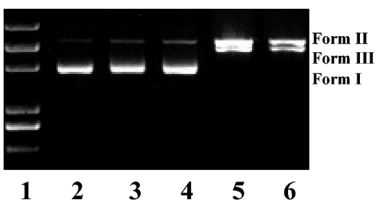

Fig. 2 Agarose gel of pUC 19 (7 μg ml^{-1}) incubated in Tris-HCl buffer (50 mM, pH 7.4) at 37 °C in the dark for 24 h. Lane 1, DNA marker; Lane 2, DNA control; Lane 3, 6 mM of EDTA; Lane 4, 0.57 mM of cyclen; Lane 5, 0.57 mM of PNA–cyclen conjugate + 6 mM EDTA; Lane 6, 0.57 mM of PNA–cyclen conjugate.

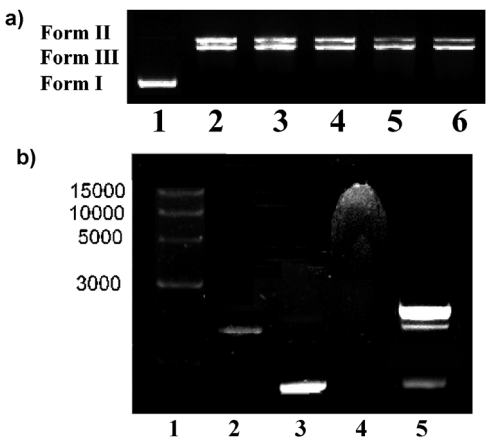

Fig. 3 (a) Agarose gel electrophoresis assays for the effect of ROS scavengers on the cleavage reaction. Lane 1, DNA control; Lane 2, 0.57 mM PNA–cyclen conjugate control; Lanes 3–6, 0.57 mM PNA–cyclen conjugate in the presence of DMSO (1 M), NaN_3 (1 M), *t*-BuOH (1 M), KI (1 M), respectively. (b) Agarose gel for ligation of pUC 19 DNA linearized by the PNA–cyclen conjugate. Lane 1, DNA markers; Lane 2, pUC 19 linearized by PNA–cyclen without T4 DNA ligase; Lane 3, DNA control; Lane 4, pUC 19 linearized by PNA–cyclen with T4 DNA ligase; Line 5, pUC 19 cleaved by PNA–cyclen conjugate.

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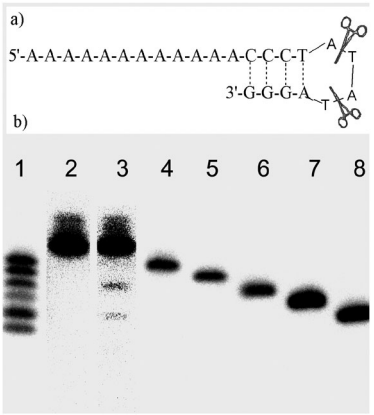

Fig. 4 (a) Schematic view of sequence-selective cleavage of 24-nt hairpin DNA by PNA–cyclen conjugate. (b) Phosphoimager picture of a 15% polyacrylamide/7 M urea gel showing the cleavage products of the oligonucleotide ^{32}P-labeled at the 5′-end. Line 1, 16–21 nt markers; Line 2, DNA alone, Line 3, as in Line 2 with the addition of PNA–cyclen conjugate (0.57 mM), Lines 4–8, 21–17 nt markers respectively.

a substrate was accordingly phosphorylated at its 5′-end with [γ-^{32}P] ATP in the presence of T4 polynucleotide kinase and further purified by PAGE. The cleavage reaction of the oligonucleotide was carried out in Tris-HCl (50 mM, pH 7.4) at 37 °C for 48 h by adding 0.57 mM PNA–cyclen conjugate. As shown in Fig. 4, two new faster moving bands were observed (Lane 3) and the new bands were identified to be 19-mer (Lane 6) and 17-mer (Lane 8), respectively, according to the weight markers. All cleavages were located inside the loop region which was in close proximity to the terminal PNA modification. While in the absence of the PNA–cyclen conjugate, no cleavage products were observed in the labeled DNA strand (Lane 2) under the same conditions. The result demonstrated the cleaving sites were in close proximity to the binding site on the DNA strand of the PNA-cyclen conjugate. However, when alterations of nucleotides in the stem (*e.g.* from A═T to T═A or G≡C to C≡G) of the oligonucleotide were made, the resulting PNA–cyclen conjugate exhibited no cleavage activity (Fig. S10, ESI†). These observations indicated that the cleavage of PNA–cyclen relies selectively on the binding sequence for its activity.

We have also examined DNA site-selective cleavage by using the MALDI-TOF analysis. A similar 12-nt hairpin oligonucleotide substrate (5′-OH-CCCTATATAGGG-3′-OH) was used and the obtained molecular masses of the cleaved fragments are shown in Fig. S11 (ESI†). Cleavage fragments at m/z = 1546, 1746, 2139 and 3092 (detailed analyses are shown in Table S1†) addressed to the products 5′-OH-ATAGGG-3′-OH binding with the PNA–cyclen conjugate ($[M + H]^+$, calc. 3092; $[M + 2H]^{2+}$, calc. 1546), 5′-OH-CCCTAT-3′-OH ($[M + H_3O]^+$, calc. 1745), and 5′-OH-CCCTATA-3′-phosphate ($[M + H_3O]^+$, calc. 2138). The results were consistent with the PAGE experiments. In addition, it was further proved that phosphodiester bonds of DNA were cleaved *via* the hydrolysis pathway. According to the results and some reports from the literature,[8b,14,15] a plausible mechanism for DNA cleavage is schematically depicted in Scheme S1.†

In summary, a water soluble PNA-based compound was designed and characterized to serve as a nuclease mimic. This new class of metal independent artificial nucleases gives reasonably site-selective hydrolysis of the DNA targets under physiological conditions. Owing to the significant sequence selectivity of PNAs, cleavage agents conjugated to PNAs have been an ongoing research area in our group, and they could regulate efficiently DNA cleavage with well-defined sequence selectivity. So far the system has displayed modest rate of cleavage, and in most cases, the cleaver has been used in excess amounts. In future experiments we will aim at finding reasonably high rate class of PNA-based artificial nucleases which could display multiple turnover of site-selective hydrolysis of the DNA targets.

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