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Chemical modifications of artificial restriction DNA cutter (ARCUT) to promote its *in vivo* and *in vitro* applications

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Keywords: invasion, molecular crowding effect, nuclear localization signal peptide, PNA, pcPNA, site-selective scission, triplex formation

Abbreviations: ARCUT, artificial restriction DNA cutter; Boc, tert-butyloxycarbonyl; EDTA, ethylenediamine-*N,N,N′,N′*-tetraacetic acid; NLS, nuclear localization signal; pcPNA, pseudo-complementary peptide nucleic acid; PNA,, peptide nucleic acid; PEG, poly (ethylene glycol); Py-Im polyamide, pyrrole-imidazole polyamide.

Recently, completely chemistry-based tools for site-selective scission of DNA (ARCUT) have been prepared by combining 2 strands of pseudo-complementary PNA (pcPNA: site-selective activator) and a Ce(IV)-EDTA complex (molecular scissors). Its site-specificity is sufficient to cut the whole human genome at one predetermined site. In this first-generation ARCUT, however, there still remain several problems to be solved for wider applications. This review presents recent approaches to solve these problems. They are divided into (i) covalent modification of pcPNA with other functional groups and (ii) new strategies using conventional PNA, in place of pcPNA, as site-selective activator. Among various chemical modifications, conjugation with positively-charged nuclear localization signal peptide is especially effective. Furthermore, unimolecular activators, a single strand of which successfully activates the target site in DNA for site-selective scission, have been also developed. As the result of these modifications, the site-selective scission by Ce (IV)-EDTA was achieved promptly even under high salt conditions which are otherwise unfavourable for double-duplex invasion. Furthermore, it has been shown that "molecular crowding effect," which characterizes the inside of living cells, enormously promotes the invasion, and thus the invasion seems to proceed effectively and spontaneously in the cells. Strong potential of pcPNA for further applications *in vivo* and *in vitro* has been confirmed.

Introduction

Man-made tools that can cut DNA selectively at predetermined sites have been nowadays attracting much interest.[1-3] Manipulation of huge genomes of higher animals and plants is one of the primary goals of these studies. Naturally occurring restriction enzymes are inappropriate for this purpose, because (i) their site-specificity is too low to cut the genomes at one site and (ii) their recognition sites are limited in number and targetable sequence. We have already developed a completely chemistry-based tool for site-selective scission of DNA.[4,5] This artificial restriction DNA cutter (ARCUT; **Fig. 1**) is composed of 2 strands of pseudo-complementary PNA (pcPNA: site-selective activator) and Ce(IV)-EDTA complex (molecular scissors). This DNA analog is easily prepared by solid-phase synthesis,[6] and very strongly binds to cDNA through Watson-Crick base-pairing.[7] Furthermore, a pair of pcPNA strands can invade double-stranded DNA to form so-called double-duplex invasion complex.[8,9] This invasion is facilitated by using pseudo-complementary nucleobases (2,6-diaminopurine D and 2-thiouracil U_s), in place of conventional A and T, and destabilizing the pcPNA/pcPNA duplex. The double-duplex invasion induces various effects on biological functions *in vivo* and *in vitro*.[10-12] In ARCUT, the binding sites of these pcPNA strands to the corresponding DNA strands are laterally shifted each other so that predetermined sites in both DNA strands remain single-stranded (see the middle in Fig. 1). These single-stranded portions are selectively cut by the Ce(IV)-EDTA complex, which hydrolyzes only single-stranded DNA, resulting in the aimed site-selective scission.[13] The site-specificity of ARCUT can be easily modulated by changing the lengths and the sequences of the pcPNAs used, and be made high enough to cut the whole human genome (3×10^9 base-pairs) at one predetermined site.[14] Many applications of this tool have been already documented. As an example, the telomere at the end of each human chromosome (e.g., Xp/Yp) was selectively clipped and compared in the length.[15] The sub-telomere region of a chromosome was cut with the use of ARCUT, and the corresponding telomere was obtained. Although the telomeric repeats are exactly the same for all the chromosomes ($(TTAGGG)_n$ in the case of human beings), the sequence in this region is specific to each chromosome and can be targeted by ARCUT. By using another ARCUT, the telomere at the end of another chromosome (e.g., 11q) was clipped. It was found that the length of telomere is different from a chromosome

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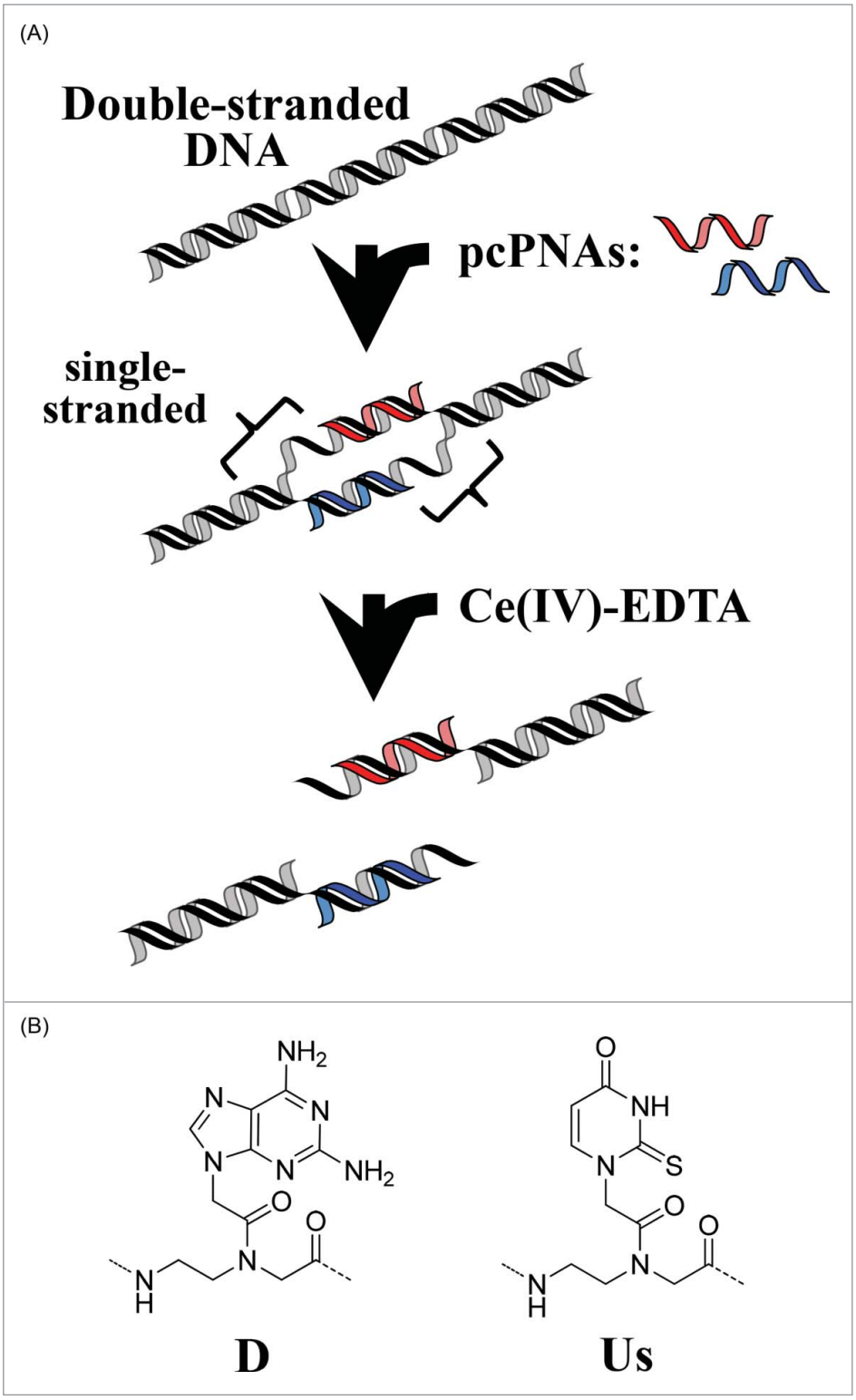

Figure 1. Schematic view of site-selective scission of double-stranded DNA by the first-generation ARCUT (combination of a pair of pcPNA + Ce(IV)-EDTA).

to chromosome even in the same cells, indicating that the telomeres have still more complicated and important roles than proposed before. Furthermore, ARCUT has opened the way to completely restriction-enzyme-free DNA manipulation. The fragments obtained by the ARCUT scission at desired site can be directly combined each other or with other fragments with the use of DNA ligase. Even when there is no recognition site of naturally occurring enzyme, required recombinant DNA is straightforwardly obtainable.[4,5] The cutters are also applicable to gene editing in human cells by cutting the genome at designated site and promoting the desired homologous recombination.[16-18]

All these results confirm that ARCUT is a useful and highly promising tool for the future biology and biotechnology. In this first-generation ARCUT, however, there still remain several problems to be solved for even wider applications. The typical ones are as follows.

1. The sequences which are targetable should be still more flexible and versatile. Although they are rather freely chosen even with the first-generation ARCUT, some sites (e.g., highly GC rich regions) are difficult to target.
2. Site-selective scission by the ARCUT is not very effective in the media containing high concentrations of salts. This disadvantage must be solved especially for *in vivo* applications, since the inside of living cells is abundant in metal ions (K^+, Na^+, Mg^{2+}, and others). It is well known that these high salt conditions are unfavorable for double-duplex invasion of unmodified pcPNAs (and thus for the site-selective scission by the ARCUT).
3. For *in vivo* applications, the cutters must be transferred to the nuclei and localized there where the genomes as target of site-selective scission exist.
4. At physiological temperatures, the invasion process is not sufficiently fast and cannot proceed spontaneously. In most of the previous experiments, the invasion complex was prepared by incubating the mixture at higher temperatures (e.g., 50°C).
5. It is more desirable if conventional (non pseudo-complementary) PNA can be used, in place of pcPNA. They are

obtainable more easily and economically, and are directly relevant to naturally occurring DNA and RNA.

It has been already evidenced that both the site-selectivity and the scission efficiency of ARCUT are primarily governed by double-duplex invasion processes, in which the hotspots for the scission (single-stranded portions) are formed in double-stranded DNA. In order to improve the functions of ARCUT and promote its applications *in vivo* and *in vitro*, these invasion processes must be contr-olled and promoted through covalent or non-covalent modification of pcPNA. This review presents recent approaches to solve these problems. Increased versatility of site-selective DNA cutters available should undoubtedly widen the scope of applications to biology, biotechnology, medicine, and others. At the same time, these improvements of the invading activity of pcPNA (and of PNA) should also greatly facilitate other applications of the invasion which have been already well documented by many laboratories.[19-36]

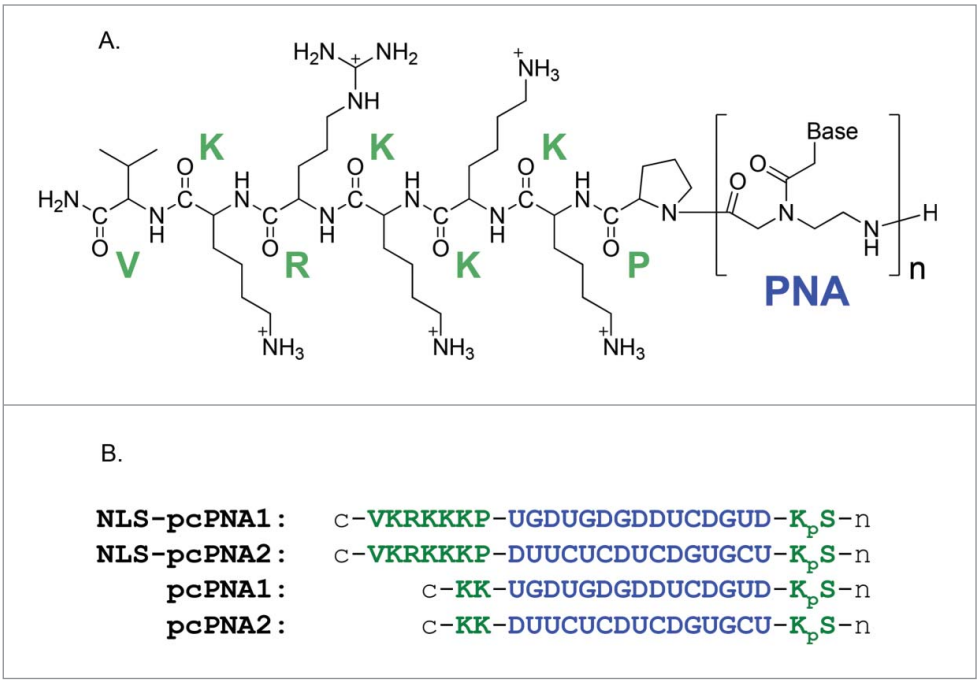

Figure 2. (**A**) Chemical structure of NLS-pcPNA conjugate. (**B**) Sequences of NLS-pcPNAs and pcPNAs. The NLS peptide was directly attached to the C-terminus of pcPNA, and all the pcPNAs have a phosphoserine ($_pS$) at the N-termini. The 2-thiouracil U_s is shown as U here.

Covalent Modifications of pcPNA to Enhance Site-Selective Scission by ARCUT

Conjugation of pcPNA with nuclear localization signal peptide[37]

As described above, double-duplex invasion of (unmodified) pcPNA prefers low salt concentrations, and hardly occurs at physiological ionic strengths.[38-42] There, double-stranded DNA is so stable that the energy loss accompanied by its dissociation cannot be sufficiently compensated by the formation of 2 pcPNA/DNA duplexes in double-duplex invasion. The stability of these hetero-duplexes is not so much affected by ionic strength. One of the most plausible strategies to solve this problem is to attach positively-charged moieties to pcPNA and stabilize these duplexed through enhanced electrostatic interactions with negatively-charged DNA.[31] Based on these arguments, the nuclear localization signal (NLS) peptide of SV40 (PKKKRKV) was attached to the C-terminus of pcPNA strand (**Fig. 2**). This heptapeptide has large positive-charges (+5) at pH 7 and function in cells as "tag" to import a protein into the nuclei. Previously, conventional (non pseudo-complementary) PNA was modified with this oligopeptide. The conjugates were efficiently introduced to the cells, transferred to the nuclei, and exhibited antigene effects.[43-48]

It was found that double-duplex invasion is greatly promoted by the conjugation of pcPNA with the NLS. The invasion of a pair of NLS-pcPNA conjugates efficiently occurred at small [conjugate]/[DNA] ratios where unmodified pcPNAs without the NLS hardly invaded the DNA (**Fig. 3**). Still more importantly, the invasion of the NLS-pcPNA is strongly resistant against ionic strength in the media. Even in the presence of 100 mM NaCl, where the invasion of unmodified pcPNA never occurred, this conjugate efficiently and promptly invaded the DNA. In **Fig. 4**, the salt concentrations were further increased up to the values in living cells: [NaCl] = 12 mM, [KCl] = 140 mM, and [$MgCl_2$] = 0.8 mM. Consistently with the previous works from many laboratories, the invasion of a pair of unmodified pcPNAs was marginal under these conditions. With the NLS-pcPNA pair, however, notable invasion occurred even when [pcPNA]/[DNA] = 4 and became further dominant with the increase in the ratio. The mismatch-discrimination ability was also kept satisfactorily high for the NLS-pcPNA. Thus, the invasion completely disappeared, when one base-pair in 20-bp target DNA sequence was changed to another base-pair. By conjugating pcPNA with the NLS, double-duplex invasion has been successfully accomplished under the intracellular salt conditions. The electrostatic interactions between the positive charges of the NLS moiety and the negative charges of DNA simply strengthen the binding affinity of NLS-pcPNA to the target DNA, and satisfactorily compensate the energy loss due to the dissociation of the DNA/DNA duplex. Furthermore, the sequence-specificity in the invasion is hardly deteriorated in spite of the promoted electrostatic interactions. Superiority of the NLS is remarkable.

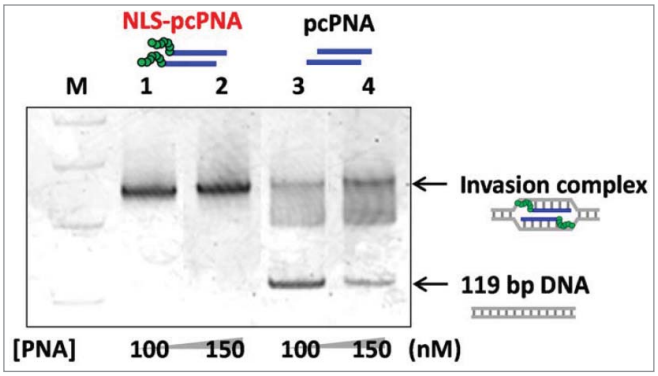

Figure 3. Gel mobility shift assay for the formation of invasion complex using pcPNAs with or without the conjugation of NLS peptide. Invasion conditions; [double-stranded DNA (119 bp)] = 50 nM, [each of NLS-pcPNAs or pcPNAs] = 100 or 150 nM and [Hepes (pH 7.0)] = 5 mM at 50°C for 1 h. Reproduced by permission from ref. 37.

In addition to the thermodynamic promotions described above, the NLS conjugation also increased the rate of invasion to significant extent. As shown in **Fig. 5**, the NLS-pcPNAs invaded the DNA promptly, and the reaction virtually completed only in about 30 min. The conversion in the plateau of the time-course (fraction of the invasion complex in the solution to the starting DNA) was around 90%. On the other hand, the invasion of unmodified pcPNAs was far slower and took more than 2 h to attain the plateau (the conversion = 35%). Thus, the NLS modification promoted the double-duplex invasion both thermodynamically and kinetically. Electrostatic attraction between the NLS and the DNA should be certainly one of the main factors responsible for these effects. However, this interaction cannot be the sole factor, since another positively-charged peptide RRRRR provides only poor results for the invasion and non-selective interactions of the conjugate with the DNA prevails there; *vide infra*. The primary structure of the NLS, its tertiary structure and/or its hydrophilicity/hydrophobicity balance should be also important.

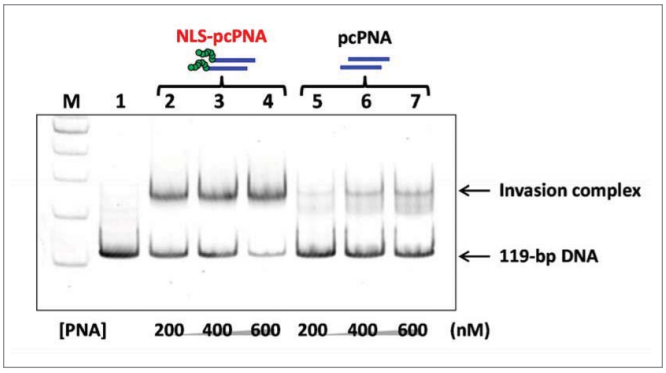

Figure 4. Invasion complex formation under intracellular salt conditions. [double-stranded DNA (119 bp)] = 50 nM, [NaCl] = 12 mM, [KCl] = 140 mM, and [$MgCl_2$] = 0.8 mM at pH 7.0 and 50°C for 2 h. Reproduced by permission from ref. 37.

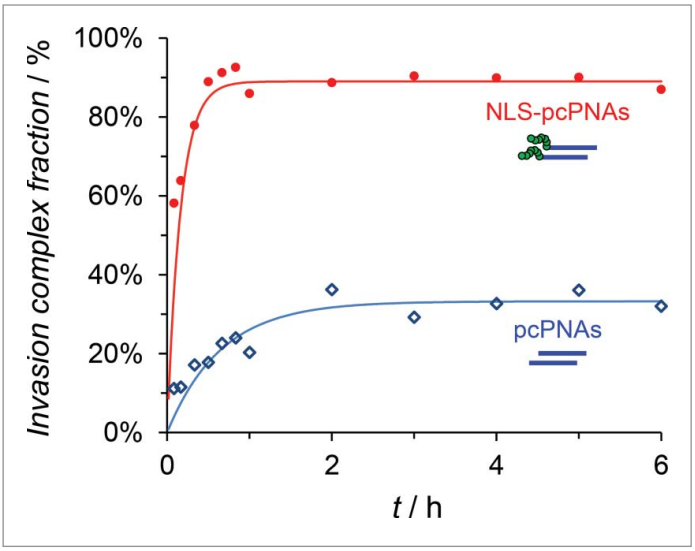

Figure 5. Time-courses of invasion-complex formation. Conditions; [double-stranded DNA (119 bp)] = 50 nM, [each of NLS-pcPNAs or pcPNAs] = 150 nM, [Hepes (pH 7.0)] = 5 mM and [NaCl] = 100 mM at 50°C. Reproduced by permission from ref. 37.

In **Fig. 6**, the effect of the NLS on Ce(IV)-EDTA-mediated site-selective scission of DNA was examined under high salt conditions. First, the substrate DNA was treated with the combination of NLS-pcPNA1 and NLS-pcPNA2 in the presence of 100 mM NaCl, and then Ce(IV)-EDTA was added. Two scission products of expected sizes (1.7 and 3.0 kbp) were formed, confirming the site-selective scission. On the other hand, a pair of unmodified pcPNAs provided no scission products under the same conditions. These results are completely consistent with the invasion experiments presented above, and clearly show the essential role of the NLS moieties in the promotion of the site-

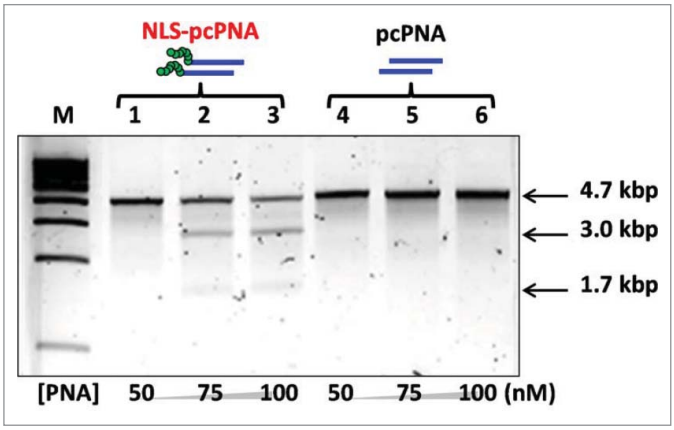

Figure 6. Site-selective scission of double-stranded DNA by artificial DNA cutters composed of NLS-pcPNAs at high NaCl concentration (100 mM) at 50°C for 16 h. The scission mixtures involve [DNA substrate (4,733 bp)] = 4 nM, [HEPES (pH 7.0)] = 5 mM, and [Ce(IV)-EDTA] = 100 μM. The invasion complexes were prepared by incubating the mixtures at 50°C for 2 h. Reproduced by permission from ref. 37.

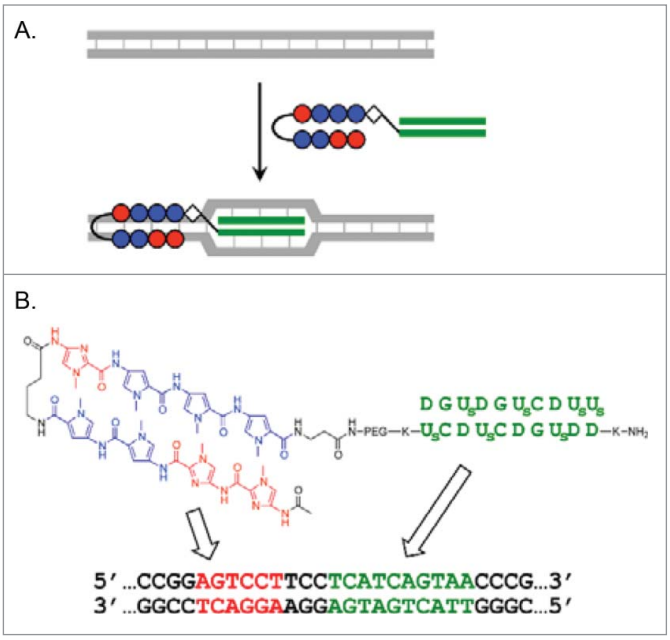

Figure 7. (**A**) Recognition of double-stranded DNA by combining the invasion of 2 pcPNA strands (decamers) and Py-Im polyamide binding (recognizing 6 bp in DNA). (**B**) Conjugate of decamer pcPNA and Py-Im polyamide. DNA sequences recognized by each portion are presented. Reproduced by permission from ref. 49.

selective DNA scission by ARCUT. Furthermore, the NLS moiety allows the conjugates to be transferred to the nuclei and preferentially localized there, promoting various *in vivo* applications.

Conjugation of pcPNA with pyrrole-imidazole polyamide[49]

In another attempt to facilitate double-duplex invasion, pcPNA was conjugated with the pyrrole-imidazole polyamide (the balls in **Fig.** 7). This Py-Im polyamide was developed by Dervan's group, and selectively binds to target site in double-stranded DNA.[50-52] The DNA binding of this polyamide is primarily based on multiple hydrogen bondings in the minor groove, and accompanies minimal structural change of the DNA. Thus, the binding successfully occurs even in the presence of high concentrations of salts. By taking advantage of this feature of Py-Im polyamide, double-duplex invasion of the conjugate and the resultant Ce(IV)/EDTA-mediated site-selective DNA cleavage were successfully accomplished under high salt conditions.

The conjugate of pcPNA3 and a Py-Im polyamide was synthesized using solid-phase synthesis. The pcPNA3 (H_2N-$DDU_sGDCU_sDCU_s$-COOH) is complementary to one strand of DNA (130 bp), whereas the Py-Im polyamide is designed to selectively bind to the 5′-AGTCCT-3′/3′-TCAGGA-5′ site in this DNA which is adjacent to the double-duplex invasion site of pcPNA3/pcPNA4. Accordingly, the combination of the conjugate and pcPNA4 (H_2N-$K_2DGU_sDGU_sCDU_sU_sK$-H) should

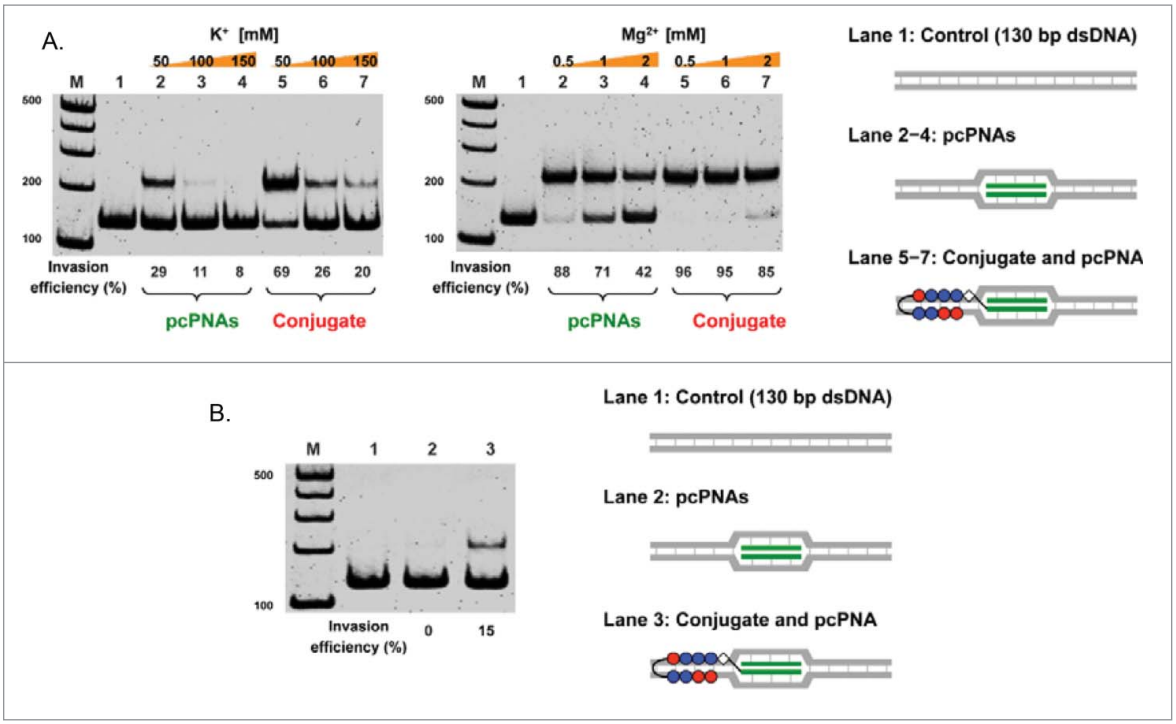

Figure 8. (**A**) Binding of DNA (130 bp) by the combination of the pcPNA3/Py-Im conjugate and pcPNA4 at higher salt conditions. Lane 1, DNA only; lanes 2–4, with pcPNA3/pcPNA4 (without the polyamide); lanes 5–7, combination of the conjugate and pcPNA4; M, 100 bp ladder. Invasion conditions: [DNA] = 10 nM, [conjugate] = [pcPNA3] = [pcPNA4] = 100 nM, [HEPES] = 5 mM at pH 7.0 and 37°C for 20 h. (**B**) Gel-shift assay for DNA-binding under physiological salt conditions ([NaCl] = 12 mM, [KCl] = 139 mM and [$MgCl_2$] = 0.8 mM). Lane 1, DNA only; lane 2, with pcPNA3/pcPNA4 combination (200 nM each); lane 3, with the conjugate/pcPNA4 combination (200 nM each). Invasion conditions: [DNA] = 10 nM at pH 7.0 and 37°C for 20 h. Reproduced by permission from ref. 49.

recognize 10 bp by the double-duplex invasion and additionally the adjacent 5 bp by the minor groove binding. Importantly, the combination efficiently bound to the DNA even in the presence of 50–150 mM KCl (**Fig. 8A**). In contrast, the binding of pcPNA3/pcPNA4 pair (without the conjugation with the Py-Im) was far weaker and greatly suppressed by the salts. The decameric pcPNAs are too short to form double-duplex invasion complexes efficiently under these conditions, and thus the strong resistance of the DNA binding by the Py-Im polyamide against metal salts is crucial here. The superior DNA binding capacity of the conjugate/pcPNA4 combination over the pcPNA3/pcPNA4 pair was also evident, when the KCl was replaced with $MgCl_2$. In the presence of 1–2 mM $MgCl_2$, most of the DNA formed the invasion complex with the combination, whereas only small portion bound to the pcPNA3/pcPNA4 pair. The mismatch recognition of the combination was also satisfactory. Upon the change of one C/G base-pair in the polyamide-binding site to A/T, the dissociation constant K_D decreased by 30 fold according to surface plasmon resonance analysis. The conjugate bound to the fully-matching DNA much faster ($k_a = 7.8 \times 10^3$ $M^{-1}s^{-1}$) than the mismatching DNA ($k_a = 8.6 \times 10^2$ $M^{-1}s^{-1}$). When the number of base-pairs between the pcPNA-binding site and the polyamide-binding site was changed, the binding efficiency was as follows: CC < TCC > GTTCC. The optimal distance was 3 base-pairs.

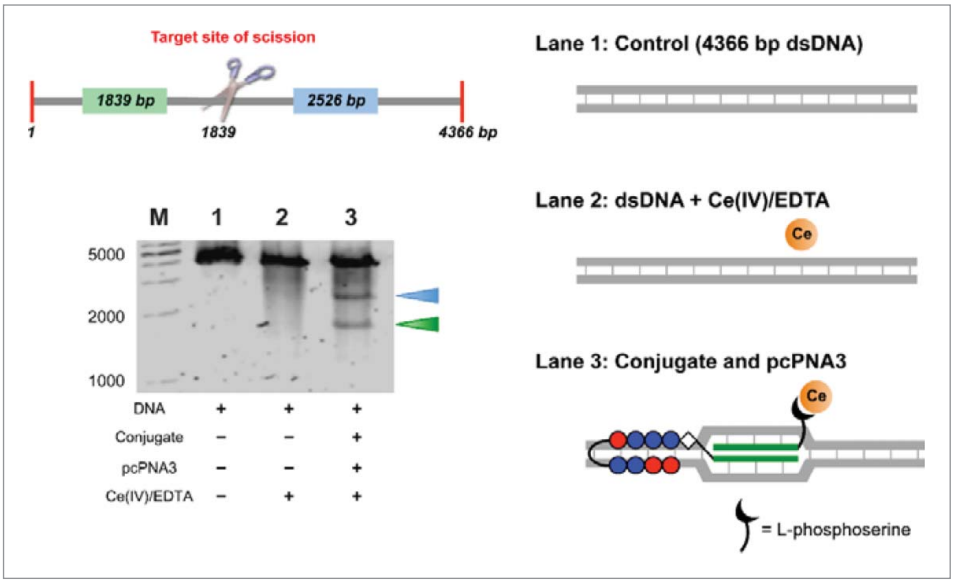

Figure 9. Site-selective hydrolysis of double-stranded DNA (4366 bp) with the pcPNA3/Py-Im conjugate and pcPNA4 combination. Lane 1, control; lane 2, with Ce(IV)-EDTA only; lane 3, with Ce(IV)-EDTA in the presence of the combination. Reaction conditions: [DNA] = 4 nM, [conjugate] = [pcPNA4] = 200 nM, [Ce(IV)-EDTA] = 200 μM, and [NaCl] = 100 mM at pH 7.0 and 50°C for 16 h. Reproduced by permission from ref. 49.

It is noteworthy that the conjugate/pcPNA4 combination successfully formed invasion complex even under intracellular salt conditions in living cells ([NaCl] = 12 mM, [KCl] = 139 mM, and [$MgCl_2$] = 0.8 mM). As shown in Fig. 8b, the invasion of this combination to the DNA was notable even under these high salt conditions in which the pcPNA3/pcPNA4 pair without the NLS modification never showed the binding. Consistently, double-stranded DNA (4,366 bp) was site-selectively hydrolyzed by combining the Py-Im polyamide-pcPNA3 conjugate with pcPNA4 and treating the system with Ce(IV)-EDTA (**Fig. 9**). Two fragments (about 1,840 and 2,530 bp) for the scission at the binding site of the combination of the conjugate and pcPNA2 were produced. The hot spots for the scission (single-stranded portions) should be formed at the invasion-site, probably through enhanced molecular breathing motion. As expected, no scission occurred in the absence of the combination.

Attachment of metal-binding ligands to pcPNA and *in situ* preparation of site-selective DNA cutter[53]

An EDTA was attached to the *N*-terminus of pcPNA with the use of a new Boc-monomer bearing *tert*-butyl-protected EDTA (the top in **Fig. 10**), and site-selective cutter of double-stranded DNA was prepared by *in situ* oxidation of EDTA-bound Ce(III) to catalytically active Ce(IV). The EDTA-pcPNA conjugates were obtained by the solid-phase synthesis, and a pair of them was incubated with DNA substrate to form the double-duplex invasion complex. Then, $Ce^{III}(NO_3)_3$ was added to the solution, and the Ce(III) was allowed to coordinate to the EDTA ligand. The DNA hydrolysis was carried out simply by incubating the mixture at 50°C under air. Although Ce(III) itself (its EDTA complex also) is virtually inactive for DNA hydrolysis, it was *in situ* converted to Ce(IV) through the oxidation by molecular O_2, and acquired the eminent catalytic activity for DNA hydrolysis.[54-58] This *in situ* preparation of site-selective DNA cutter was experimentally confirmed by the results in Fig. 10a. Only the DNA cutter, prepared from $Ce(NO_3)_3$ and a pair of EDTA-pcPNA conjugates (not from $Ce(NO_3)_3$ + a pair of pcPNA strands without EDTA), showed site-selective hydrolysis. In the site-selective DNA cutter prepared with this methodology, catalytically active Ce(IV) is bound to the EDTA and strictly located there, and thus its effective concentration for the catalysis is sufficiently large. Accordingly, the site-selective DNA scission can be still more clear-cut than the first-generation ARCUT, in which the site-selectivity for the scission is based on the difference in the activity for DNA hydrolysis (single-stranded DNA vs. double-stranded DNA). The pcPNA bearing an iminodi(methylphosphonic acid) (bisP), in place of EDTA, was also effective for this *in situ* preparation of site-selective DNA cutter (Fig. 10b).

This new methodology is reminiscent of *in situ* preparation of DNA cutters which selectively hydrolyze single-stranded DNAs.[59] There, a multiphosphonate ligand (nitrilotris (methylenephophonate) or *N,N,N,N*-ethylenediamine tetra

(methylenephosphonate)) was bound to the ends of oligonucleotides. Using two of these modified oligonucleotides, a gap-structure was formed at the targeted scission-site in single-stranded DNA substrate so that the ligands were placed at the gap-site. Then, $Ce(NO_3)_3$ was added to the solution and the reaction was carried out under air. In the reaction mixture, most of the Ce(III) ions were bound to the multiphosphonate ligand, and converted by *in situ* oxidation to catalytically active Ce(IV) in the complex. Thus, the amount of unbound Ce(IV) in the reaction mixture, which could lead to undesired off-target scissions, was minimized. As the result, the scission by these cutters was so strictly restricted to the gap-site that single-stranded DNAs, even of considerable length (e.g., 800 base-length), were cut at the designated site with a very high selectivity. The scission fragments were successfully used to prepare recombinant DNAs.

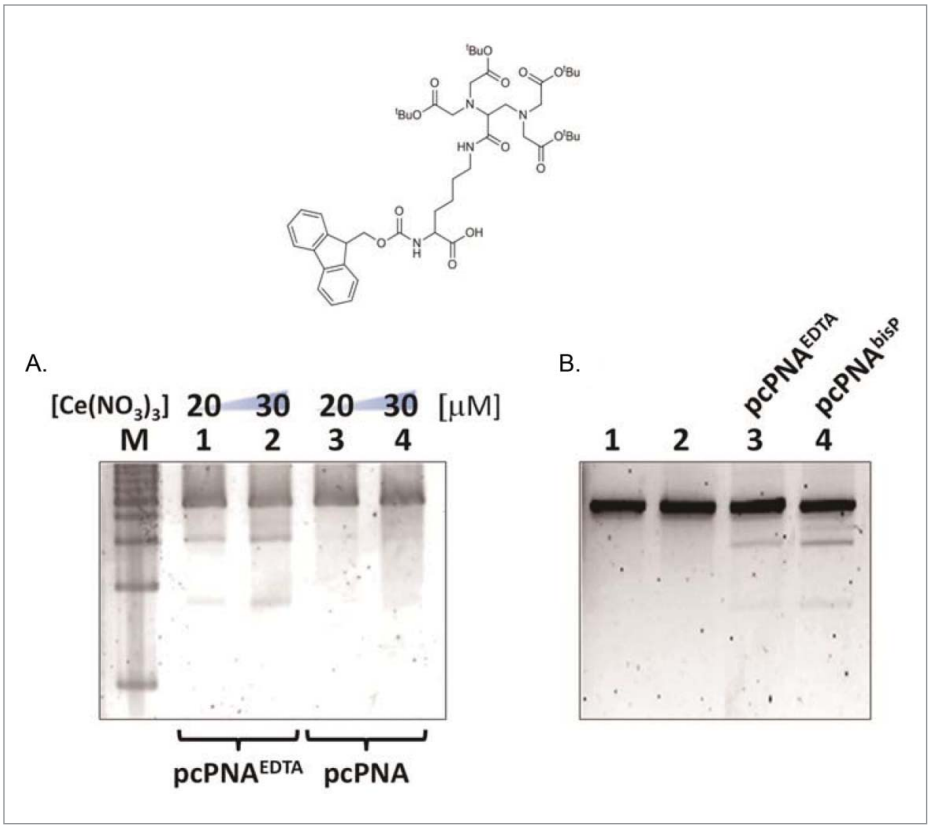

Figure 10. Site-selective scission of DNA by pcPNAs using the cutters prepared by *in situ* oxidation of Ce (III). (**A**) DNA scission with EDTA-bearing pcPNA. Lanes 1 and 2, pcPNA-5^{EDTA}/pcPNA-6^{EDTA}; Lanes 3 and 4, pcPNA-5/pcPNA-6. Lane M, 1,000 bp ladder. Reaction conditions: [DNA] = 4 nM, [each pcPNA] = 100 nM, [NaCl] = 100 mM at pH 7.0 and 50°C for 17 h under air. The conjugates were prepared using the monomer presented at the top of this figure. (**B**) Comparison of the effect of EDTA ligand with bisP ligand ([Ce $(NO_3)_3$] = 30 μM). Lane 1, DNA only; Lane 2, + Ce(III) only; Lane 3, pcPNA-5^{EDTA}/pcPNA-6^{EDTA} + Ce(III); Lane 4, pcPNA-5^{bisP}/pcPNA-6^{bisP} + Ce(III). In all the conjugates, the ligand was attached to the *N*-terminus of pcPNA. Reproduced from ref. 53.

Note that, in all other works described in this review, the source of Ce(IV) is the tetravalent salt $Ce^{IV}(NH_4)_2(NO_3)_6$, rather than the trivalent salts (e.g., $Ce^{III}(NO_3)_3$). Accordingly, the activation process by the oxidation with molecular oxygen is never required to obtain the DNA cutters, and the site-selective scission can be straightforwardly and successfully achieved even in anaerobic conditions such as the inside of cells.

Use of Conventional (Non Pseudo-Complementary) PNA as Site-Selective Activator in ARCUT

Site-selective activation of double-stranded DNA by one strand of the conjugate between conventional PNA and nuclear localization signal peptide (NLS)[60]

Even with the first-generation ARCUT, selective-scission site can be rather freely chosen. But still GC-rich regions are not very easy to be targeted, since the promotion of double-duplex invasion by pseudo-complementary bases D and U_s, used for naturally occurring bases A and T, is one of the primary factors for effective function of this cutter. These two modified bases sterically repel each other and destabilize the duplex between the 2 pcPNA strands which are intrinsically complementary with each other. On the other hand, they form stable base-pairs with the corresponding counterpart bases A and T in DNA. Thus, pcPNA/DNA duplexes are sufficiently stable, allowing the efficient formation of the invasion complex. However, appropriate pseudo-complementary bases for G and C, which strongly bind to natural bases C and G but hardly form a mutual base-pair, have not yet been fully developed.[61-63] Thus, in GC-rich regions of substrate DNA, there is no efficient way to destabilize the corresponding pcPNA/pcPNA duplex and promote the invasion. One of the direct solutions to this problem is to develop a methodology in which only one pcPNA strand can activate the targeted site in double-stranded DNA. Here, conventional PNA involving only A, G, C, and T nucleobases can be directly used, since pcPNA/pcPNA duplexes are never formed. In fact, single PNA strand notably invaded double-stranded DNA when target site had a special sequence (e.g., inverted repeats) and in addition positively-charged amino acids or oligopeptides were attached to the PNA.[31] Chemical modification of the backbone with gamma PNA, as well as of nucleobases with G-clamp nucleobase, was also effective for the purpose.[39,41]

On the basis of these findings, it was recently found that single strand of the PNA-NLS conjugate (**Fig. 2A**) successfully activates the target site in plasmid DNA (importantly, conventional PNA

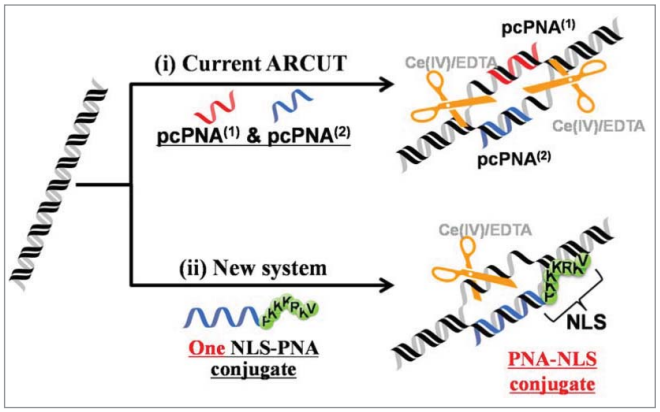

Figure 11. Schematic representations of (**i**) site-selective DNA cutter using 2 pcPNA strands (the first-generation ARCUT) and (**ii**) new DNA cutter using only one strand of NLS-attached conventional PNA. The NLS was directly attached to the C-terminus of conventional PNA bearing no pseudo-complementary bases. Reproduced by permission from ref. 60.

involving no pseudo-complementary bases was used there). Under the conditions where an unmodified PNA strand never binds to the DNA, a strand of this conjugate notably invades the DNA. The hot spot, formed by this single-molecular activator, was selectively hydrolyzed by Ce(IV)-EDTA (**Fig. 11**). Targeted scission sequences can be rather freely chosen. First, a sequence (16 bp) in exon 2 of *N-myc* oncogene (*MYCN*) was incorporated into a plasmid DNA, and targeted by an NLS-PNA1/Ce(IV)-EDTA system (**Fig. 12A**). The NLS was directly attached to the C-terminus of 16-mer PNA1, which is complementary to the sense-strand (T340-A355) of *MYCN*. It should be noted that PNA1 involves only conventional nucleobases and no pseudo-complementary bases are used. This target sequence is highly GC rich (7 consecutive G-C pairs) and almost random without inverted repeat. When the PNA1 binds to the sense-strand according to the Watson-Crick rule, the corresponding anti-sense-strand becomes single-stranded. The site-selective hydrolysis of DNA (3,563 bp) by Ce(IV)-EDTA was carried out at pH 7.0 and 37°C. The single-stranded portion, formed by the unimolecular invasion, was hydrolyzed, converting the form I DNA (supercoiled) to the form II (relaxed open-circular), and then to the form III (linear). In order to pin down the scission site, the reaction mixture was further digested with a restriction enzyme Sma I, and the whole products were analyzed by agarose gel electrophoresis. Only two scission bands were observed, confirming the site-selective scission. The sizes of these 2 bands were exactly identical to those (around 1.5 and 2.0 kbp) expected from the double digestion by the NLS-PNA1/Ce(IV)-EDTA system and Sma I. The universal nature of this single-molecular activation was further confirmed by the selective scission of another 16 bp sequence in blue fluorescence protein (BFP) (**Fig. 12B**). The scission by the NLS-PNA2/Ce(IV)-EDTA system (followed by digestion with Xba I) provided 2 fragments corresponding to the expected dual scission. It was concluded that only one strand of NLS-PNA conjugate is sufficient to activate the target site in the plasmid DNA and induces the site-selective scission by Ce(IV)-EDTA. As was the case in the first-generation ARCUT,[64] mismatch-discrimination ability of this system was satisfactory, and the scission was completely gone upon the change of one base-pair in the targeted scission sequence to another. When a strand of unmodified PNA was used as the activator, in place of the corresponding NLS conjugate, no DNA scission occurred.

The critical importance of the NLS group for the present site-selective scission is definitely confirmed by the fact that the conjugate with another positively charged peptide (RRRRR) never functioned as unimolecular site-selective activator. When PNA-RRRRR conjugate was combined with Ce(IV)-EDTA, non-targeted sites were notably cut and the scission at the target site was never a dominant process. Apparently, this non-NLS conjugate bound to the DNA rather randomly through electrostatic interactions and activated various sites in this DNA. The primary structure of the NLS, its tertiary structure and/or the hydrophilicity/hydrophobicity balance would be important.

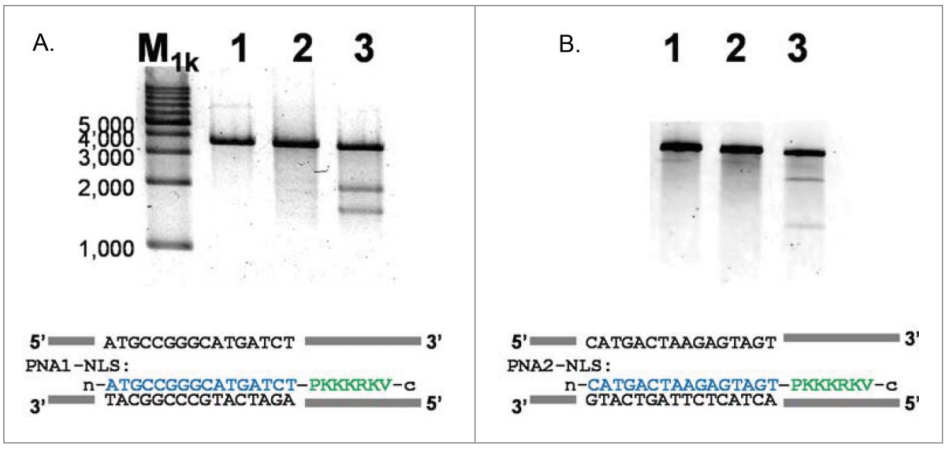

Figure 12. Site-selective DNA scission by combining one strand of PNA-NLS conjugate and Ce(IV)-EDTA. In (**A**) and (**B**), a 16-bp sequence in the exon 2 of *MYCN* gene and *BFP* gene in a plasmid was targeted, respectively. Lane 1, DNA only (control); Lane 2, DNA treated with Ce(IV)-EDTA (control); Lane 3, the scission with the combination of Ce(IV)-EDTA and PNA-NLS conjugate. Reaction conditions: [DNA] = 4.0 nM, [PNA-NLS] = 200 nM, [Ce(IV)-EDTA] = 200 μM, and [NaCl] = 100 mM at pH 7.0 and 37°C for 3 d The scission mixture was treated with Sma I (for **A**) or with Xba I (for **B**) before the electrophoresis. Reproduced by permission from ref. 60.

Invasion complex formation of the NLS-PNA conjugate with the target plasmid was further confirmed by fluorescence spectroscopy. A Quasar 570 dye was covalently bound to the N-terminus of NLS-PNA2 conjugate. The emission efficiency of this dye is primarily governed by the *cis-trans* isomerization in the photo-excited singlet state, and the quantum yield of this photo-isomerization sharply depends upon both the micro-environmental freedom and the interactions with adjacent aromatic residues.[65] It was found that the Dye-NLS-PNA2

conjugate emitted notable fluorescence only in the presence of the plasmid. The decay profiles were also remarkably affected by the plasmid (**Fig. 13**). All the decay curves were composed of 2 exponential terms, indicating the presence of 2 photo-excited singlet states. Without the plasmid, the shorter lifetime term (τ_1 = 0.14 ns) was the major component (A_1 = 82%, the bottom line). In its presence, however, the longer lifetime component (τ_2 = 1.72 ns) became dominant (A_2 = 73%, the top line). Upon the formation of invasion complex, the Quasar 570 dye is placed in a sterically restricted field and also in the vicinity of many nucleobases. Accordingly, its *cis-trans* photo-isomerization is notably suppressed, resulting in the increases in both the fluorescence intensity and the lifetime. Consistently, the complementary oligonucleotide showed smaller but similar effects on the fluorescence (the middle curve). Previously reported antigene effects of NLS-PNA conjugates for cancer treatments could be associated with this type of unimolecular invasion to the genome.[44-48]

As described in the section 1.1, a pair of the conjugates of pcPNA with the NLS efficiently invades double-stranded DNA. Thus, these conjugates show both double-duplex invasion and single-molecular invasion. Comprehensive study on the stability of these 2 kinds of invasion complexes and the other properties has not yet been achieved.

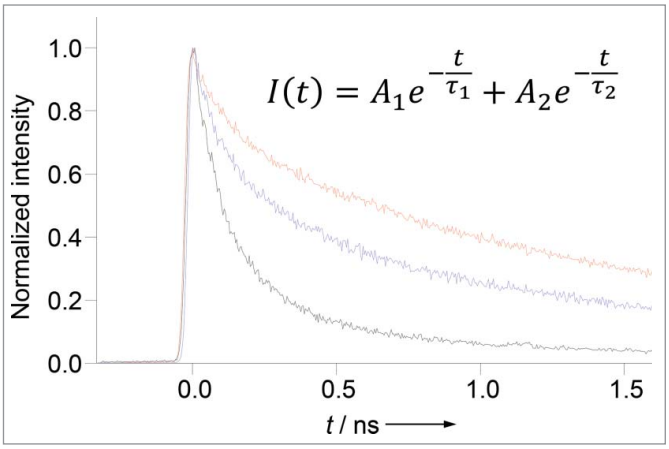

Figure 13. Fluorescence decay curves of the Quasar 570 dye-bound PNA2-NLS conjugate (excitation, 395 nm; photon counting, from 550 nm to 620 nm). Bottom, Dye-PNA2-NLS alone; top, Dye-PNA2-NLS + the plasmid. The values of τ1 (s), τ2 (s), A1, and A2, obtained by fitting the curves, are (0.14, 0.91, 0.82, 0.18) and (0.18, 1.72, 0.27, 0.73), respectively. For the purpose of comparison, the result for the 16-mer complementary oligonucleotide is shown by the middle curve. Measurement conditions are [Dye-PNA2-NLS] = 500 nM and [DNA] = 0 or 1000 nM at pH 7.0. Reproduced by permission from ref. 60.

Site-selective DNA scission by combining a triplex-forming bis-PNA with Ce(IV)-EDTA[66]

Homopyrimidine/homopurine sequences take crucial roles in a range of cell functions (e.g., transcriptional regulation, chromatin organization, and DNA repair),[67,68] and are also proposed to be the binding sites of non-coding RNA for transcriptional regulation.[69-71] For the site-selective scission of these sites, the first-generation ARCUT is practically inapplicable, since pcPNA strands involving consecutive purine sequences are very difficult to prepare by solid-phase synthesis (in most cases, the coupling efficiency significantly decreases with increasing product length). However, these sequences were successfully targeted by combining triplex-formation of PNA with Ce(IV)-EDTA (**Fig. 14**). Two PNA strands were connected with appropriate linker to enhance the triplex-forming activity, and used as unimolecular site-selective activator (these kinds of conjugates are termed as bis-PNA).[72,73] Advantageously, only one PNA conjugate involving conventional PNA (containing no pseudo-complementary bases) is required to activate a predetermined site for the site-selective scission. Upon the triple-helix formation, single-stranded portion was formed in one DNA strand and selectively cut by Ce(IV)-EDTA. This primary scission in turn promoted the hydrolysis of the counterpart strand of the DNA, resulting in site-selective scission of both strands of the double-stranded DNA substrate.

In **Fig. 15**, a 16 bp homopyrimidine/homopurine sequence in human chromosome 4 (5′-CTCTCTCTCTCTTTCC-3′/3′-GAGAGAGAGAGAAAGG-5′), being related to Hutchington's disease, was chosen as a target for the site-selective scission. In bis-PNA1, 2 homopyrimidine PNA strands (composed of 16 nucleobases) were connected by a poly(ethylene glycol) linker (*PEG*). The sequences of 2 PNA strands were designed on the basis of the formation of TAT and C^+GC triplets. There is no need to use pseudo-complementary nucleobases, and thus conventional PNA can be employed. The combination of bis-PNA1 and Ce(IV)-EDTA successfully showed site-selective DNA scission of plasmid DNA which involved the target 16 bp sequence. When a 10 bp region (5′-CTCTCTTTCC-3′/3′-GAGAGAAAGG-5′) in plasmid was targeted with the use of a shorter bis-PNA2, site-selective DNA scission was also successful in

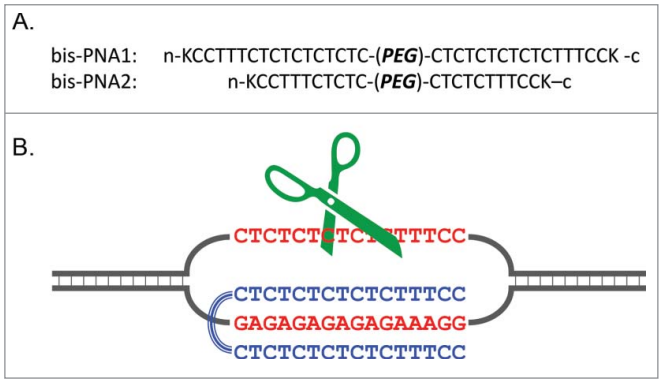

Figure 14. Triple-helix forming bis-PNAs (**A**) and site-selective scission of double-stranded DNA by combining bis-PNA with Ce(IV)-EDTA (**B**). The single-stranded homopyrimidine sequence (the upper strand of DNA in (**B**) is hydrolyzed by the Ce(IV)-EDTA, and this primary scission promotes the hydrolysis of the counterpart homopurine sequence. The linker portion (*PEG*) is a consecutive linkage of 3 residues of 8-amino-3,6-dioxaoctanoic acid and K stands for a lysine residue. Reproduced by permission from ref. 66.

terms of the same strategy. The sites which are difficult to target by the first-generation ARCUT have been successfully cut.

Invasion Complex Formation Under Molecular Crowding Conditions[74]

In spite of a number of extracellular applications of pcPNA invasion, its intracellular applications have been rather limited, primarily because of the following 2 experimental results (note that they were obtained under non-molecular crowding conditions). First, the inside of cells is abundant in metal ions and thus seems to be inappropriate for the invasion. Second, the invasion is a rather slow process at physiological temperatures, and does not proceed spontaneously there (or takes a very long time for its completion). Although targeted mutation was successfully corrected with the use of a pair of pcPNAs in living cells,[75] details on the behaviors of these pcPNAs in the cells (whether they really formed the invasion complex or not) still remained unknown.

However, it has been confirmed that neither of these 2 factors appears to be critical for the applications in living cells, since "molecular crowding effect" [76,77] significantly facilitates the double-duplex invasion. The inside of cells is largely occupied by proteins and other biomolecules, and thus its physicochemical properties are much different from bulk aqueous phase. In the study, the influence of these specific intracellular environments ("molecular crowding effect") was simulated by the addition of polyethylene glycol (PEG) to aqueous solutions.[78,79] It was found that PEG greatly promotes double-duplex invasion of pcPNA, and allows its formation at high salt conditions where otherwise the invasion never occurs. As an example, PEG of averaged molecular weight 200 (PEG200) was added to pH 7.0 Hepes buffer containing 100 mM NaCl (**Fig. 16**). The two pcPNA strands (H-K-$U_sU_sDCU_sGDU_sGD$-KK-NH_2 and H-K-$U_sCDU_sCDGU_sDD$-NH_2) have 10 nucleobases. Without the addition of PEG200 ([each pcPNA] = 100 nM), the invasion never occurred at 37°C under the conditions employed. When PEG200 was added to this medium, however, the double-duplex invasion was remarkably promoted and notable amounts of invasion complexes were formed (lanes 2 and 3). Similar invasion-promoting effects were also observed for PEGs of other degrees of polymerization. When one mismatch was introduced between each pcPNA and its corresponding DNA strand by changing an A/T pair in the substrate DNA to G/C pair, no double-duplex invasion complex was detectable even in the presence of 20 % or 40 % (w/v) PEG200 (lanes 5 and 6). Mismatch-recognition

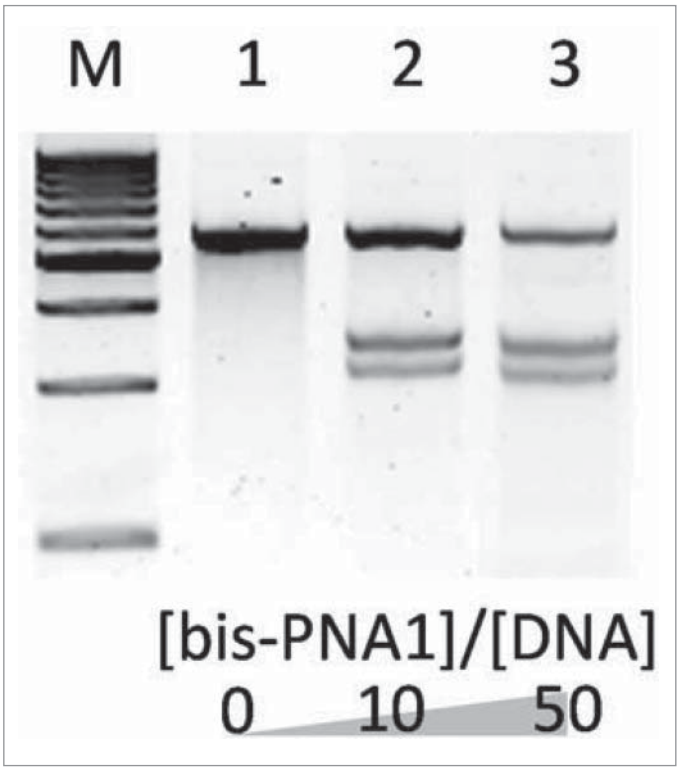

Figure 15. Site-selective scission of DNA (2685 bp) by combining triplex-forming bis-PNA1 and Ce(IV)-EDTA at the [bis-PNA1]/[DNA] ratios of 0 (lane 1), 10 (lane 2), and 50 (lane 3). Lane M, 500 bp ladder. Conditions; [DNA] = 4 nM, [bis-PNA1] = 40–200 nM, [Ce(IV)-EDTA] = 100 μM, and [NaCl] = 100 mM at pH 7.0 and 50°C for 16 h. Reproduced by permission from ref. 66.

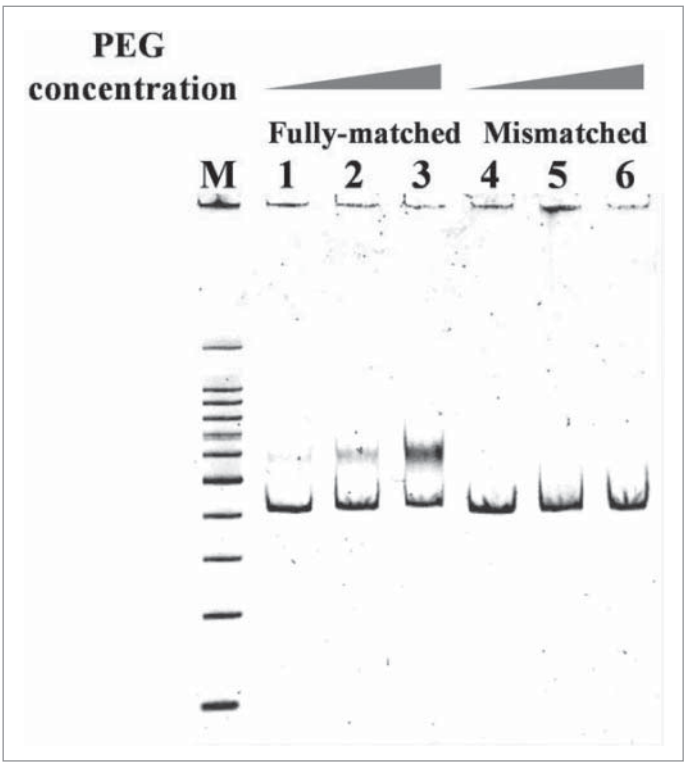

Figure 16. Effects of molecular crowding conditions on the invasion complex formation of pcPNA with double-stranded DNA. [PEG200] = 0 (lanes 1 and 4), 20 (lanes 2 and 5), and 40 w/v % (lanes 3 and 6), respectively. The invasion conditions are [DNA (408-bp)] = 5 nM, [each pcPNA] = 100 nM, and [NaCl] = 100 mM at pH 7.0 (Hepes buffer) and 37°C for 1 h. In lanes 4–6, one base-pair in the DNA at the invasion site was changed from A/T to G/C, and one mismatch was introduced between the DNA strand and each of the pcPNAs. Lane M: 100 bp markers. Reproduced by permission from ref. 74.

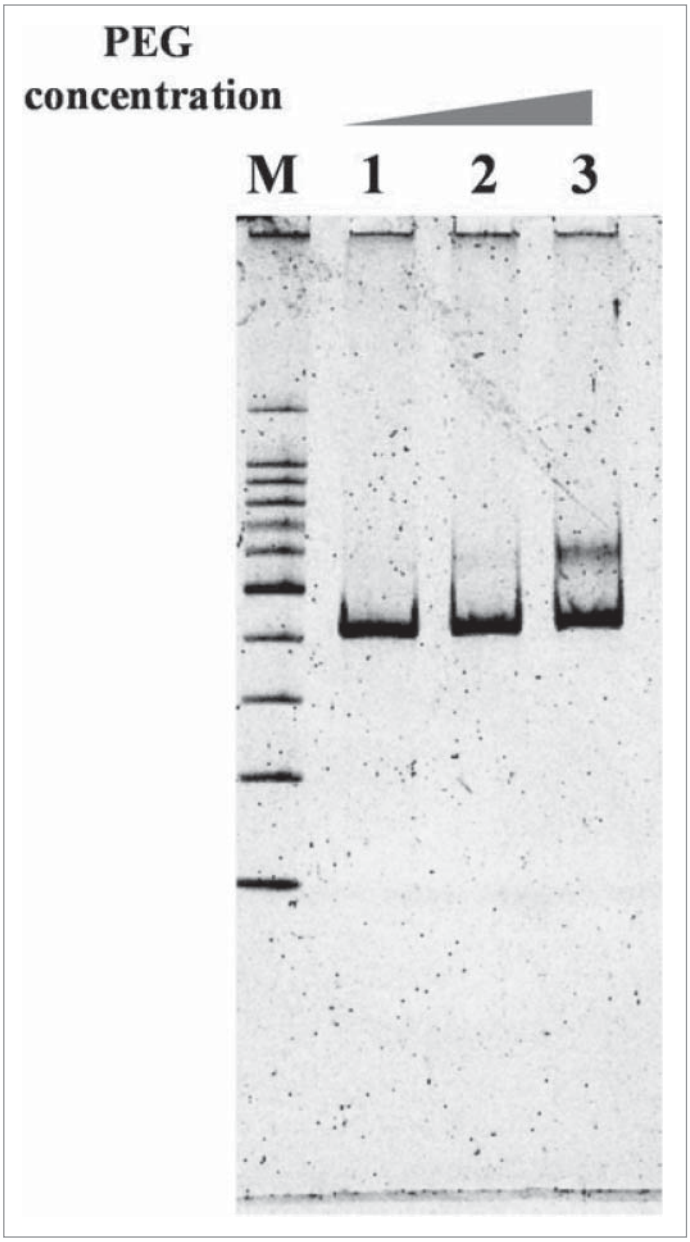

Figure 17. Invasion complex formation of pcPNA with double-stranded DNA in the medium mimicking the inside of living cells ($[K^+] = 140$ mM, $[Na^+] = 10$ mM, and $[Mg^{2+}] = 0.5$ mM) under the molecular crowding conditions. [PEG200] = 0 (lane 1), 20 (lane 2), and 40 w/v % (lane 3). Other invasion conditions are the same as **Fig. 16**. Reproduced by permission from ref. 74.

under molecular crowding conditions was satisfactory. According to Sugimoto et al.,[80,81] both DNA/DNA duplex and PNA/DNA duplex are destabilized by molecular crowding effect, primarily due to the decrease of the activity of water in the media. However, the magnitude of destabilization of DNA/DNA duplex by this effect is far larger than the magnitude of destabilization of the corresponding destabilization of PNA/DNA duplex. As the result, double-duplex invasion is promoted by the molecular crowding effect. The destabilization of the DNA/DNA duplex under molecular crowding conditions should also facilitate the breathing motion of the duplex and be favorable for spontaneous formation of the invasion complex.

Even under the high salt conditions in the inside of living cells ($[Na^+] = 10$ mM, $[K^+] = 140$ mM, and $[Mg^{2+}] = 0.5$ mM), the invasion complex was formed to notable extent (**Fig. 17**). Importantly, the whole process of the double-duplex invasion spontaneously occurred at 37°C, and the rate was fast enough to complete the process at least within 1 h. This is highly in contrast with the fact that the double-duplex invasion under non-molecular crowding conditions was never a spontaneous process, and accordingly these complexes were usually prepared by incubating the components at higher temperatures (e.g., 50°C) for several hours. In these preparations, the salt concentrations in the media were in most cases kept minimal without adding any salts except for the buffer reagents. All these results substantiate that pcPNAs, incorporated into the living cells by some means, should easily bind to the target site in the genome through double-duplex invasion, and exhibit designated functions there.[10-12] Strong potential of pcPNA for further intracellular (and *in vivo*) applications has been confirmed.

Conclusions

Site-selective DNA cutter (ARCUT), previously developed by our group, is composed of 2 strands of pcPNA and Ce(IV)-EDTA complex.[4,5] The single-stranded portions, formed in both strands of double-stranded DNA through double-duplex invasion of the pcPNA, are hydrolyzed by Ce(IV)-EDTA. Recently, it has been shown that covalent and non-covalent modifications of pcPNA can greatly promote the double-duplex invasion and thus the resultant site-selective DNA scission. Some of the confronting problems of the first-generation ARCUT (the problems (1)-(4) presented in the introduction section) have been solved. Site-selective activation of double-stranded DNA by a single PNA strand has been also developed to provide new types of site-selective DNA cutters (solutions to the problems (1) and (5)). Furthermore, "molecular crowding effect," which characterizes the specific environments in cells, has been shown to enormously facilitate the double-duplex invasion and allow successful invasion inside the living cells. It is indicated that the problems (2) and (4) are not critical when the pcPNA invasion is practically used *in vivo* for various applications. All these results show that double-duplex invasion of pcPNA (as well as ARCUT based on this invasion) should be highly promising for future applications in biochemistry, biology, medicine, and biotechnology.

In addition to these chemistry-based DNA cutters, protein-based DNA cutters have been also recently devised. Typical examples are conjugates of the nuclease domain of *Fok*I restriction enzyme with either zinc finger proteins (ZFN[82]) or transcription activator-like effectors (TALEN[83,84]). Homing endonucleases recognizing very long cognate sequences are engineered to target desired sites.[85] RNA-guided site-selective scission of genome by a nuclease (CRISPR-Cas system) has been also reported.[86,87] These cutters can be produced in living cells by transfecting the corresponding DNA vectors and used for

intracellular gene editing. On the other hand, chemistry-based DNA-cutters have a remarkable feature that they can be freely modulated, according to our need, through chemical modification. Thus, required functions can be easily provided. This flexibility of molecular design is one of the most significant advantages of these cutters. Apparently, both chemistry-based DNA cutters and protein-based DNA cutters have pros and cons, and should be complementarily used for further developments of the relevant fields.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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