



Notes & Tips

Targeting linear duplex DNA with mixed-base peptide nucleic acid oligomers facilitated by bisPNA openers

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PD-loop complexes¹ formed by bisPNA openers and a mixed-base (mb) oligonucleotide on double-stranded (ds) DNA enable various diagnostic and biotechnological manipulations with linear DNA duplexes [1–5]. So far, the PD-loop formation requires the use of two homopyrimidine bisPNA oligomers to open the DNA duplex for binding an oligonucleotide [6]. This condition imposes certain sequence limitations on the PD-loop-forming sites. Besides, substantial overlap between the bisPNA and oligonucleotide sequences is an unavoidable feature of PD-loops. Indeed, only those oligonucleotides that are longer than 10 nt can form sufficiently stable hybridization complexes within the PD-loops, whereas for their efficient formation the peptide nucleic acid (PNA) openers cannot be separated by more than 10 bp [6].² Such sequence overlap necessitates the sequential dsDNA targeting first by PNA openers with subsequent removal of unbound bisPNAs followed by targeting by an oligonucleotide. Otherwise, PNA openers would obstruct the oligonucleotide binding to dsDNA if targeted simultaneously and/or without removal of unbound PNA oligomers.

These two major requirements on the PD-loop formation limit their applications. Here we demonstrate that PP-loops, i.e., complexes similar to PD-loops but formed by

only PNA oligomers, are free from both of these conditions: PP-loops can form (i) with only one PNA opener and (ii) without any sequence overlap between participating oligomers. In addition, we found that in the case of PP-loops bisPNA openers can be separated, in contrast to PD-loops, by a substantially longer random DNA sequence. We assume that all of this becomes possible due to a significantly higher affinity of mbPNAs as compared with corresponding oligonucleotides [7]. Considering that PP-loops can substitute the PD-loops in some applications, our findings expand the practical applicability of the PD-loop technology.

Fig. 1 shows the schematics of dsDNA sites we targeted by mbPNAs with the aid of short bisPNA openers. First is the case when [8 + 8]-mer bisPNA1 was used to assist in the binding of 13-mer mbPNA1 to a target dsDNA fragment of the pPL3 plasmid. In this case (Fig. 1A), there is a 5-bp overlap between PNA-binding sites to facilitate the mbPNA strand-invasion binding to the corresponding dsDNA site partly preopened by bisPNA. Based on the recent discovery that binding of mbPNAs to dsDNA can occur efficiently at the very end of DNA duplexes [8], we hypothesized that a DNA duplex boundary generated at the mbPNA1-targeted site by the preceding binding of bisPNA1 may act as a starting point for the strand invasion-binding of the mbPNA, thereby resulting in the PP-loop with a single PNA opener. We also supposed that the mbPNA1-complementary, 5-bp terminal sequence of the ssDNA loop exposed by bisPNA1 would act as a hook for mbPNA1 to additionally support the strand invasion of this PNA.

The gel shift data presented in Fig. 2A demonstrate that mbPNA1 does not bind to dsDNA by itself: lanes 1 and 4 show no difference in the mobility of the target DNA fragment upon the addition of mbPNA1. However,

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¹ Abbreviations used: PD-loop, looped complex formed inside double-stranded DNA by bisPNAs and an oligonucleotide; bisPNA, “clamp” of two pyrimidine peptide nucleic acid oligomers connected by a flexible linker; mb, mixed-base; ds, double-stranded; PNA, peptide nucleic acid; PP-loop, complex similar to the PD-loop but with mbPNA used instead of an oligonucleotide; ss, single-stranded.

² If two PNA openers bind dsDNA far (>10 bp) from each other, the two DNA strands between them can form a stable duplex (see our results below), and this will prevent the binding of DNA oligonucleotide.

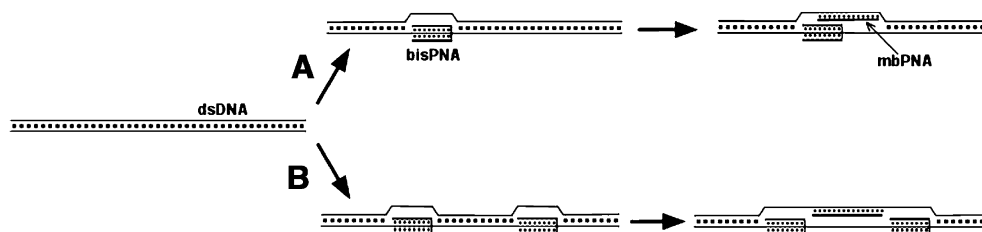


Fig. 1. Schematics of the dsDNA targeting with mbPNA oligomers facilitated by bisPNAs (shown as clamps). (A) The case where PP-loop forms with the use of a single PNA opener. Here PNAs are added sequentially to dsDNA target because of the bisPNA–mbPNA sequence overlap. (B) The case where there is no overlap between bisPNA and mbPNA sequences. Here PNAs are added to dsDNA target simultaneously (the succession of binding events, not the order of PNA addition, is shown in the figure).

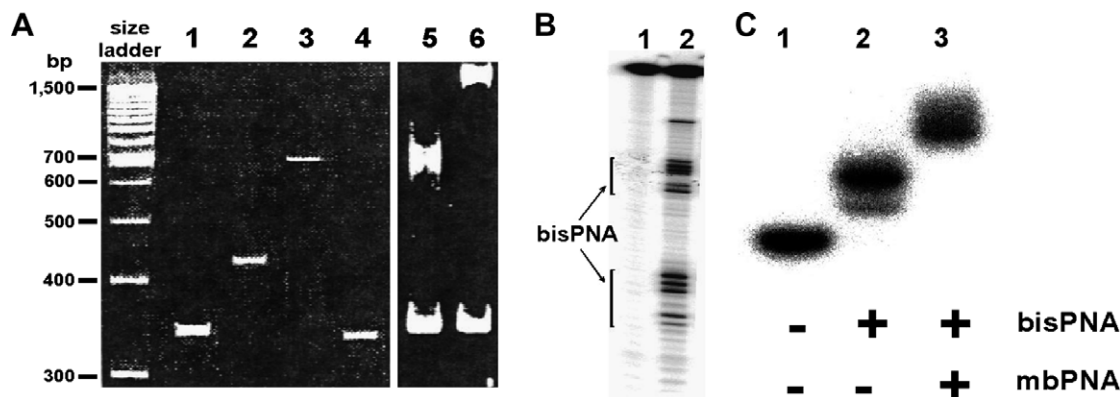


Fig. 2. (A) Gel shift assay of the complex formation between the pPL3 fragment (lane 1) and bisPNA1 (lane 2), bisPNA1 plus mbPNA1 (lane 3), and mbPNA1 alone (lane 4). Lanes 5 and 6 show the bisPNA1–mbPNA1 complex with (lane 6) and without (lane 5) streptavidin. Materials and methods: bisPNA1, H-Lys₂-T₃JT₂J₂-(eg1)₃-C₂T₂CT₃-Lys-NH₂; mbPNA1, biotin-(eg1)₃-Lys₂-GA₂G₂T₂CGA₂G₂-Lys₂-NH₂ (P. E. Nielsen's lab, Copenhagen University). The pPL3 plasmid [6] was digested by the *Pvu*II restriction enzyme to yield a 340-bp dsDNA target fragment, which carries the binding sites 5'-*AAAGAAGGTTTCGAAGG* for bisPNA1 (italic) and mbPNA1 (complement to the underlined sequence). This dsDNA fragment (0.5 pmol) was incubated with 1 μM bisPNA1 for 2 h at 37 °C in 25 mM Mes buffer (pH 6.0). Unbound PNA was removed by gel filtration of 20-μl samples through G-25 microspin columns (Amersham). Then 1 μM mbPNA1 was added to the bisPNA1-targeted samples, and they were incubated at 37 °C for another 2 h. To one of these samples, 1 μg of streptavidin (Sigma) was added. Samples were resolved in 7.5% native PAGE and were stained with ethidium bromide. (B, C) Autoradiography analysis of dsDNA binding with bisPNA2 and mbPNA2. Panel B shows a sequencing-gel analysis of the OsO₄-produced strand breaks in the p48op plasmid fragment (lane 1) and its complexes with bisPNA2 (lane 2). Positions of the target sequences for bisPNA2 are marked with brackets. Panel C shows gel shift assay of the complex formation between the p48op fragment (lane 1) and bisPNA2 (lane 2) and bisPNA2 plus mbPNA2 (lane 3). Materials and methods: bisPNA2, H-Lys₂-T₂C₂T₃-(eg1)₃-T₃C₂T₂-Lys-NH₂; mbPNA2, H-TAG₂TAT(CT₂)ATCT-Lys₃-NH₂ (Applied Biosystems). As a target for bisPNA2 and mbPNA2, the pUC19-derivative plasmid p48op was constructed, which carries the binding sites 5'-*AAGGAAATAGTTATCTCTATCTAAGGAAA* for bisPNA2 (italic) and mbPNA2 (complement to the underlined sequence). The ³²P-end-labeled *Eco*RI-*Hind*III 95-bp fragment of p48op (0.2 pmol) was mixed with 1 μM bisPNA2 alone or together with 1 μM mbPNA2 in 30 mM NaAc buffer (pH 5.0). The 15-μl mixtures were incubated at 37 °C for 6 h. In chemical probing experiments, the labeled fragment of p48op or its complexes with PNAs were reacted with OsO₄ as described previously [9]. These samples were analyzed in 8% denaturing PAGE. In gel shift assay, 12% native PAGE and 30 mM NaAc running buffer were used.

if bisPNA1 is prebound to this DNA fragment (see the shifted band in lane 2 of Fig. 2A), mbPNA1 can form stable PP-loop complex with dsDNA, as revealed by an additional decrease in the DNA duplex mobility in lane 3 relative to lane 2 of that figure. To further prove that the extra shifted band in lane 3 indeed corresponds to the complex of mbPNA1 with dsDNA, we took advantage of the fact that mbPNA1 carries biotin, a high-affinity streptavidin-binding ligand. Thus, there should be further retardation of the mbPNA1–dsDNA complex during gel electrophoresis after the addition of streptavidin. Lanes 5 and 6 in Fig. 2A prove that this was really the case; one can see the significant streptavidin-caused retardation of the dsDNA fragment bound with mbPNA1 (but not the PNA-free DNA fragment). This latter demonstration of stable formation of the PP-loop–streptavidin complex is

significant for the prospective use of PP-loops to selectively capture specific dsDNA duplexes [6].

Note that because of the complementarity of their terminal sequences of five nucleobases (due to a chosen 5-bp overlap between the PNA-binding sites), bisPNA1 and mbPNA1 would obstruct their binding to dsDNA if targeted simultaneously. Therefore, they can be targeted only sequentially—first bisPNA1 and then mbPNA1—and the nonbound bisPNA1 must be removed from the solution before adding the mbPNA1. These conditions complicate the procedure and result in a notable loss of the final PNA–DNA complex, as can be seen from the gradually decreased intensities of bands in lanes 1–3 in Fig. 2A. Hence, schemes of bisPNA-assisted mbPNA targeting to dsDNA without an overlap between PNA-binding sequences would be advantageous.

The question of whether an overlap between PNA-binding sites is absolutely necessary in the case of a single bisPNA opener assistance to mbPNA binding, and/or the extent to which such an overlap may stabilize and accelerate the mbPNA–dsDNA complex formation, requires further investigation. On the one hand, the data of Kushon and coworkers [10] showed that invasion of an mbPNA into short DNA hairpins was indeed facilitated by binding on the part of PNA oligomer to an ssDNA loop. On the other hand, the data of Smolina and coworkers [8] demonstrated that there was no substantial difference in the capturing efficiency of dsDNA fragments with and without single-stranded (ss) overhangs when these fragments were tagged by and captured with mbPNA due to its invasion into linear DNA duplexes at their termini.

Taking all of this into consideration, we next studied the dsDNA target without an overlap between PNA-binding sequences. In this case, a pair of identical bisPNA openers was chosen to secure the mbPNA binding to duplex DNA. Fig. 1B shows the schematics of dsDNA targeting when a bisPNA oligomer was employed to open both sides of the mbPNA-binding site. Here [7 + 7]-mer bisPNA2 was used as an opener, and 15-mer mbPNA2 was targeted to the random sequence of dsDNA between two septipyrimidine sequences. The absence of any significant complementarity between bisPNA and mbPNA we used in this case allows their simultaneous targeting to dsDNA, thereby simplifying and expediting the PNA-targeting protocol. Note that mbPNA2 has an arbitrary sequence with low purine content, as compared with the purine-rich mbPNA1. This feature results in a slightly decreased DNA-binding affinity of mbPNA2, thereby yielding its somewhat lower strand-invading potential [11,12].

Fig. 2B shows the results of the chemical probing experiment with osmium tetroxide, an ssDNA-sensitive, pyrimidine-reactive reagent. These data demonstrate that bisPNA2, when targeted to a target dsDNA fragment of the p49op plasmid, opens two corresponding 7-nt dsDNA sites that border on the 15-nt mbPNA2-binding site (cf. lanes 1 and 2 in Fig. 2B). The latter site remains closed, however, because it features no osmium tetroxide reactivity, thereby precluding this particular dsDNA fragment from forming the PD-loop.³ Nevertheless, the gel shift experiment shown in Fig. 2C proves that mbPNA2 can form a stable PP-loop complex with the target dsDNA fragment (evidently by strand invasion, as is shown schematically in Fig. 1B) if assisted by joint binding of bisPNA2

(when used alone, no binding of mbPNA2 was observed [data not shown]).

The results of this study extend the range of dsDNA sequences that can be targeted by PNAs. Because bisPNA openers can stably invade linear DNA duplexes at homopyrimidine dsDNA sequences as short as 5 bp [13], our findings significantly soften sequence limitations on the dsDNA targeting by PNA oligomers, thereby extending their diagnostic potential. The demonstrated possibility of the dsDNA targeting simultaneously with mbPNA oligomer and bisPNA opener(s) is also important for prospective gene-targeting PNA applications in vivo.

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³ DNA oligonucleotide normally is taken at micromolar concentrations, so an oligonucleotide cannot displace the competing DNA strand because its local concentration is much higher (it can be estimated as 1 mM)⁴. Compared with oligonucleotides, binding of mbPNA to ssDNA is much stronger [7]; therefore, mbPNA readily displaces the competing DNA strand even if the latter is in excess.

⁴The length of the extended 15- to 20-nt ssDNA is approximately 100 Å, so a single molecule (or $1/6 \times 10^{-23}$ mol) may occupy an average volume of approximately $(100 \text{ Å})^3 = 10^{-21}$ L, which corresponds to approximately 1 mM concentration.