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Sequence-Unrestricted, Watson–Crick Recognition of Double Helical B-DNA by (*R*)-MiniPEG-γPNAs

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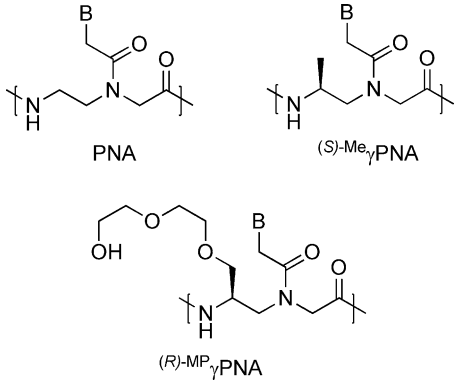
Development of general principles for designing molecules to bind sequence specifically to double-stranded DNA (dsDNA) has been a long-sought goal of bioorganic chemistry and molecular biology.[1–3] Pursuit of this goal, in the past, has generally been focused on the minor and major grooves—in large part, because of the ease of accessibility of the chemical groups that reside on these external parts of the double helix and the precedence for their recognition in nature.[2] It was long recognized that while Watson–Crick (W–C) base-pairing provides a more direct and specific means for establishing sequence-specific interactions with nucleic acid biopolymers, such as DNA and RNA, it would be difficult to do so with intact double helical DNA because of the preexisting base pairs.[4] This effort has so far led to the development of several major classes of antigene molecules, with the likes of triplex-forming oligonucleotides,[5–7] minor-groove binding polyamides,[8–11] and major-groove binding zinc-finger peptides.[12–16] While they can be designed to bind sequence specifically to dsDNA, there are still remaining issues with sequence selection, specificity and/or target length that have not yet been completely resolved,[8,13,17–19] although some progress has been made in recent years.

Over the past two decades, peptide nucleic acids (PNAs),[20] a particular class of nucleic acid mimics comprised of a pseudopeptide backbone (Scheme 1 A), have been shown to be capable of invading dsDNA.[21] This finding is significant because, contrary to the traditional belief, it demonstrates that the DNA double helix is relatively dynamic at physiological temperatures, and that W–C base-pairing interactions can be established with intact dsDNA. Though promising as antigene reagents, because of the specificity of recognition and generality in sequence design, PNA binding is presently limited to mostly homopurine[22] and homopyrimidine targets.[23] Mixed-sequence PNAs have been shown to be capable of invading topologically constrained supercoiled plasmid DNA,[24–27] conformationally perturbed regions of genomic DNA[28] and duplex termini;[29] however, they are unable to invade the interior regions of double helical B-form DNA (B-DNA)—the most stable form of DNA double helix. "Tail-clamp"[30,31] and "double-duplex invasion"[32] strategies have subsequently been developed and have enabled mixed-sequence PNAs to invade B-DNA, but they are not without limitations.[33]



Scheme 1. Chemical structures of: A) PNA, B) L-alanine-derived γPNA ($^{(S)\text{-Me}}$γPNA), and C) (*R*)-MiniPEG-containing γPNA ($^{(R)\text{-MP}}$γPNA). See ref. [36] for the synthesis of $^{(R)\text{-MP}}$γPNA monomers; the methyl ether protecting group of miniPEG side-chain is removed in the final cleavage/deprotection step of oligomer synthesis.

Recently we showed that mixed-sequence PNAs, when pre-organized into a right-handed helix by installing an (*S*)-Me stereogenic center at the γ-backbone (Scheme 1 B) can invade B-DNA.[34] However, all of our studies so far have been limited to a few selected sequences due to the poor water solubility and propensity of these first-generation $^{(S)\text{-Me}}$γPNAs to aggregate and adhere to surfaces and other macromolecules, including DNA, in a nonspecific manner. This problem is exacerbated

Table 1. Oligonucleotides employed in this study.

Oligonucleotide	Sequence	Length	G/C [%]
PNA1	H-K-GACCACAGATCTAAG-K-NH_2	15	47
$^{(S)\text{-Me}}$γPNA1	H-K-GACCACAGATCTAAG-K-NH_2	15	47
$^{(R)\text{-MP}}$γPNA1	H-K-GACCACAGATCTAAG-K-NH_2	15	47
$^{(R)\text{-MP}}$γPNA2a	H-K-CAGATCTAAG-K-NH_2	10	40
$^{(R)\text{-MP}}$γPNA2b	H-K-CCACAGATCTAAG-K-NH_2	13	46
$^{(R)\text{-MP}}$γPNA2c[a]	H-K-GACCACAGATCTAAG-K-NH_2	15	47
$^{(R)\text{-MP}}$γPNA2d	H-K-GAGACCACAGATCTAAG-K-NH_2	17	47
$^{(R)\text{-MP}}$γPNA2e	H-K-TATGAGACCACAGATCTAAG-K-NH_2	20	40
$^{(R)\text{-MP}}$γPNA3	H-K-ATTTAATAATATAAT-K-NH_2	15	0
$^{(R)\text{-MP}}$γPNA4	H-K-CTAAACTAATGTAAT-K-NH_2	15	20
$^{(R)\text{-MP}}$γPNA5	H-K-GATTACATAGATGTC-K-NH_2	15	33
$^{(R)\text{-MP}}$γPNA6	H-K-TGCGTGAGCATCAGT-K-NH_2	15	53
$^{(R)\text{-MP}}$γPNA7	H-K-CAGTGTCACGCACGG-K-NH_2	15	67
$^{(R)\text{-MP}}$γPNA8	H-K-CGGACGCAGGCTCGC-K-NH_2	15	80
$^{(R)\text{-MP}}$γPNA9	H-K-CGCCCGCCGCCCGCC-K-NH_2	15	100
$^{(R)\text{-MP}}$γPNA8b	H-K-GCGAGCCTGCGTCCG-K-NH_2	15	80
$^{(R)\text{-MP}}$γPNA8bm	H-K-CGGACGCACGCTCGC-K-NH_2	15	80

a] $^{(R)\text{-MP}}$γPNA2c and $^{(R)\text{-MP}}$γPNA1 are the same oligonucleotides, and $^{(R)\text{-MP}}$γPNA8 and $^{(R)\text{-MP}}$γPNA8b are complementary to one another; K: L-lysine.

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with increasing G/C content, making it difficult to characterize their invasion properties over a wide concentration range and broad sequence space. To rectify this problem, we replaced the methyl group with diethylene glycol (or "MiniPEG", MP; Scheme 1C). MP was chosen because of its relatively small size and hydrophilic nature, and because it has been shown to be effective in imparting water solubility and biocompatibility to nonbiological systems.[35] Our initial study revealed that inclusion of MP indeed significantly improves the water solubility and biocompatibility of PNAs,[36] but whether these MP-modified γPNAs ($^{(R)\text{-MP}}$γPNAs) can invade B-DNA has not yet been determined. Herein, we show that $^{(R)\text{-MP}}$γPNAs can invade any sequence of double helical B-DNA, ranging from 0 to 100% G/C content, with the recognition occurring in a highly sequence specific manner through Watson–Crick base pairing.

To assess binding, we performed gel-shift assays, and compared $^{(R)\text{-MP}}$γPNA1 to $^{(S)\text{-Me}}$γPNA1 and PNA1 (Table 1). A 291 bp DNA fragment containing an internal binding site was chosen as target (Figure 1A, **PM**) because initial studies had shown that this is short enough to impart electrophoretic separation upon binding of γPNAs and long enough not to interfere with the invasion efficiency (Figure S1 in the Supporting Information). The target was incubated with different concentrations of oligonucleotides in sodium phosphate (10 mM; NaPi) buffer for 16 h, followed by electrophoretic separation and staining with SYBR-Gold for visualization. Our results show that not only could $^{(R)\text{-MP}}$γPNA1 bind dsDNA, but it did so with greater efficiency than $^{(S)\text{-Me}}$γPNA, as evidenced by the relative intensity of the retarded bands (Figure 1B; compare lanes 5–7 to lanes 8–10, respectively). The dissociation constant (K_d) of $^{(R)\text{-MP}}$γPNA1 to **PM** was determined to be $3.7(\pm 0.2)\times 10^{-7}$ M. However, under identical conditions, no binding was observed for the achiral PNA (lanes 2–4). This result is consistent with the earlier finding.[37] Even at a high (100:1) oligonucleotide-to-DNA strand ratio, at which point all DNA incubated with $^{(S)\text{-Me}}$γPNA (lane 7) and PNA1 (lane 4) had disappeared presumably due to aggregation and nonspecific binding of $^{(S)\text{-Me}}$γPNA and PNA to DNA, the presence of a large excess of $^{(R)\text{-MP}}$γPNA1 had no effect on the mobility or intensity of the retarded band (lane 10). This result highlights the importance of MP at the γ-backbone in suppressing aggregation and nonspecific binding. (Table 1)

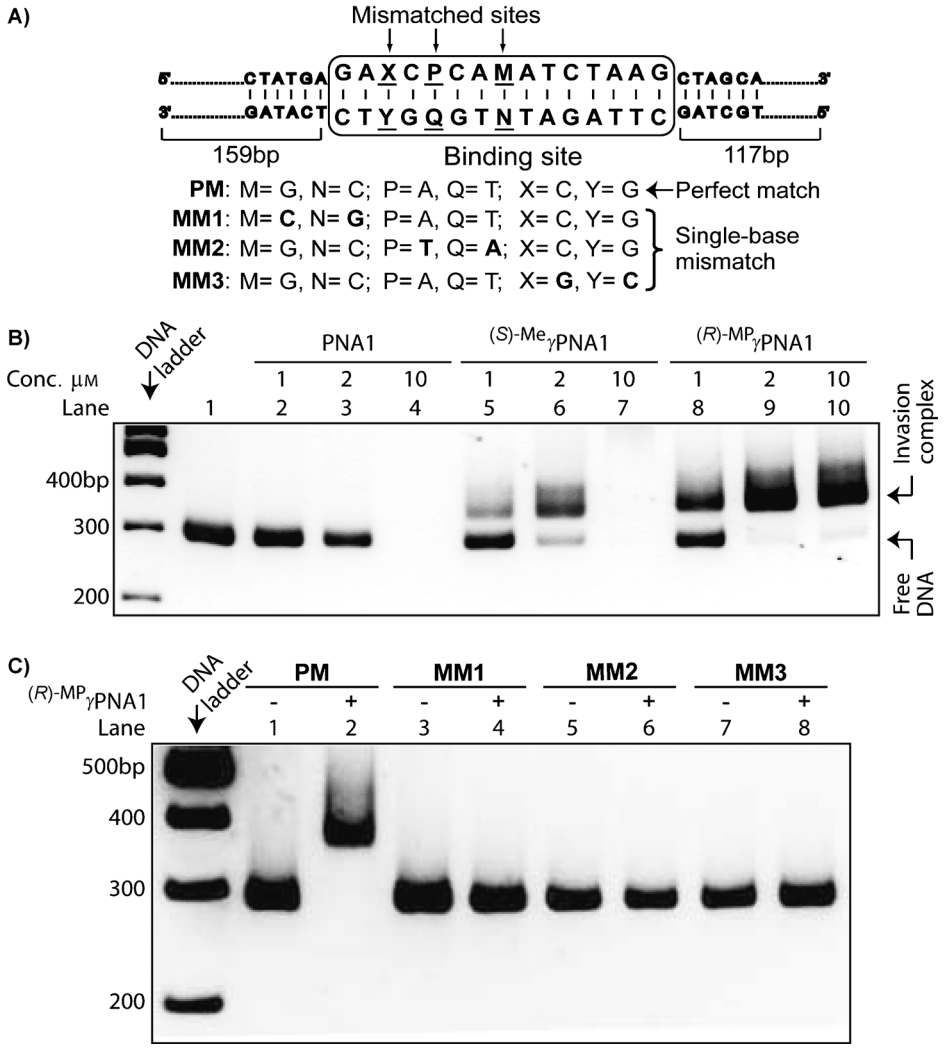

Figure 1. Effects of backbone modification and target sequence on binding efficiency. A) Schematic of the DNA targets. Results of the gel-shift assays following incubation of 0.1 μM DNA containing: B) **PM** binding site with different concentrations of PNA1, $^{(S)\text{-Me}}$γPNA1, and $^{(R)\text{-MP}}$γPNA1, and C) **PM**, **MM1**, **MM2**, and **MM3** binding sites with 2 μM $^{(R)\text{-MP}}$γPNA1 in 10 mM NaPi buffer at 37 °C for 16 h. The samples were separated on 10% nondenaturing PAGE gel and stained with SYBR-Gold.

Formation of the complex, in this case, occurred in a highly sequence-specific manner. No incubation of DNA, whether containing a single-base mismatch in the middle (**MM1**) or towards one end (**MM2** or **MM3**) resulted in formation of the retarded band (Figure 1C). This finding was further corroborated by DNase-I footprinting, which revealed protection at the expected binding site (Figure 2A). The footprinting pattern was only observed with the perfect match (lanes 2–4) and not with the single-base mismatches (lanes 5 and 6). The binding mode of $^{(R)\text{-MP}}$γPNA1 was confirmed by diethyl pyrocarbonate (DEPC)-chemical probing assay (Figure 2B),[38,39] which revealed selective cleavage at the adenine and, to a smaller extent, guanine sites on the homologous DNA strand, directly across from the binding site, following piperidine treatment. This result is consis-

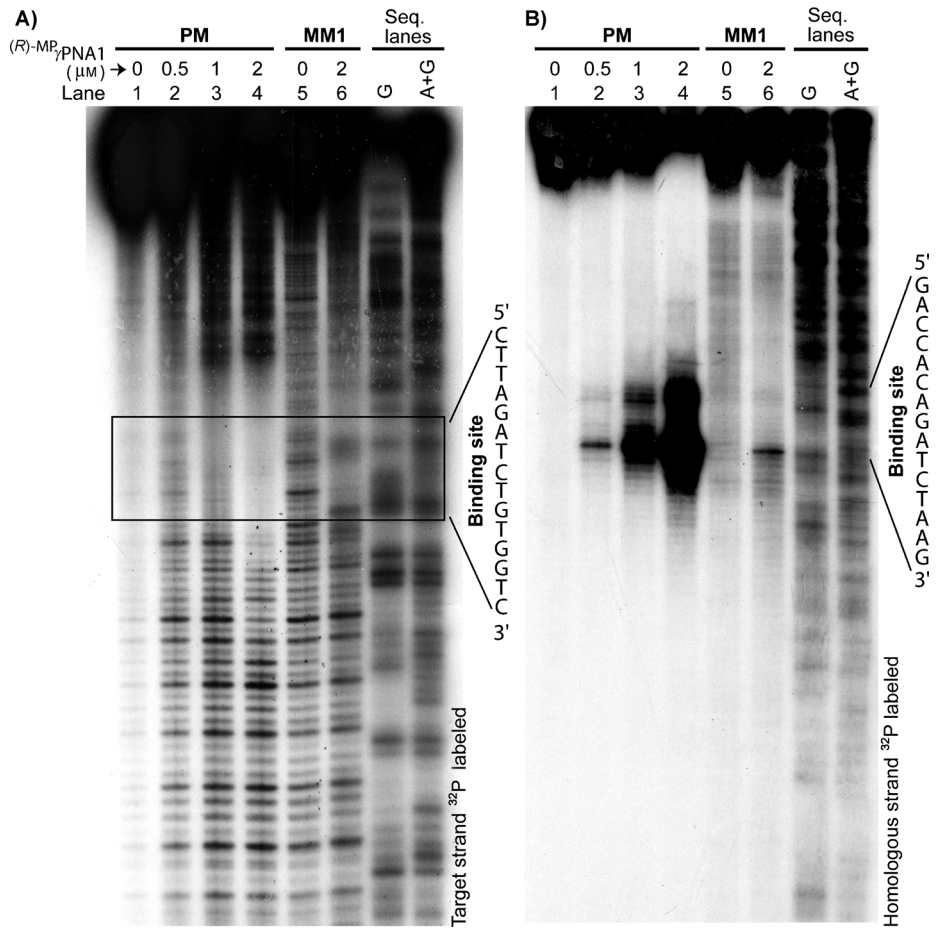

Figure 2. Results of: A) DNase-I footprinting; the target strand was labeled with ^{32}P at the 3′ end; B) DEPC-chemical probing assays following incubation of 171 bp DNA fragments containing **PM** and **MM1** binding sites with different concentrations of $^{(R)\text{-MP}}\gamma$PNA1 in 10 mM NaPi buffer at 37 °C for 16 h; the homologous strand was labeled with ^{32}P at the 3′ end.

tent with binding occurring through a strand invasion mechanism.

Next, we determined the effects of temperature and ionic strength on the invasion efficiency. Our results show that the invasion efficiency is strongly dependent on temperature (Figure 3A). This was an expected finding, because temperature has a direct effect on the rate of base-pair breathing (or opening). Strand invasion was extremely inefficient at 4 °C, in which case less than 10% of the invasion complex was formed after 24 h incubation. The rate of strand invasion dramatically increased at 37 °C and reached equilibrium within approximately 2 h. The rate obeys pseudo-first-order kinetics, with $k_{ps\,37\,^\circ C} = 0.025\ min^{-1}$ (Figure S2 in the Supporting Information)—roughly four times higher than that of $^{(S)\text{-Me}}\gamma$PNA1 for the same target.[34] Further increase in temperature resulted in additional improvement in the rate of strand invasion, but the rate increase was less pronounced from 37 to 50 °C than from 22 to 37 °C. This result indicates that at physiological temperatures, the DNA double helix is sufficiently dynamic to permit strand invasion provided that the required binding free energy can be met. Similarly, we found that the rate of strand invasion was strongly dependent on ionic strength (Figure 3B). No binding was observed at 100% physiologically simulated ionic strength (2 mM $MgCl_2$, 150 mM KCl, 10 mM sodium phosphate; pH 7.4)[40] after 16 h incubation at 37 °C. The lack of productive binding, in this case, is not due to the lack accessibility—the inability of $^{(R)\text{-MP}}\gamma$PNA1 to gain access to the nucleobase targets, but rather due to the lack of binding free energy—as demonstrated in a recent study.[41] Once bound, the complex dissociated rather slowly, with a half-life of approximately 2 h (Figure 3B, inset). This result indicates that it should be possible to perform in vitro experiments under physiologically simulated conditions with the prebound complex, as long as the experiments can be carried out within this time frame.

To assess the generality of $^{(R)\text{-MP}}\gamma$PNA binding, we determined the effects of oligonucleotide length and sequence composition on the invasion efficiency. Our results show that although a decamer, $^{(R)\text{-MP}}\gamma$PNA2a, was unable to invade dsDNA; addition of just three nucleotides ($^{(R)\text{-MP}}\gamma$PNA2b) restored the binding (Figure 4A, lane 3) and the efficiency gradually increased with increasing oligonucleotide length (lanes 4 to 6). However, we do not expect this trend to continue indefinitely, because, at some point, intermolecular $^{(R)\text{-MP}}\gamma$PNA interactions would predominate, resulting in a gradual decrease in the invasion efficiency with further increase in oligonucleotide length. We do not consider the failure of short $^{MP}\gamma$PNAs to invade dsDNA to be a detriment to most biological applications, because targeting a unique site within a mammalian genome would statistically require a recognition site of approximately 17 nucleotides in length,[42] which is within the recognition repertoire of $^{(R)\text{-MP}}\gamma$PNAs, and, if necessary, these oligonucleotides could be further chemically modified to provide the necessary binding free energy.[37,41,43]

Interestingly, we observed that unlike PNAs, even with the latest design which still requires a minimum of 40% A/T content,[33] $^{(R)\text{-MP}}\gamma$PNAs can invade any sequence of B-DNA, ranging from 0 to 100% G/C content (Figure 4B). Formation of these complexes occurred in a highly sequence specific manner. Neither cross-incubation of these oligonucleotides with other DNA targets (Figure S3 in the Supporting Information) nor incubation of the G/C-rich oligonucleotides ($^{(R)\text{-MP}}\gamma$PNA8,

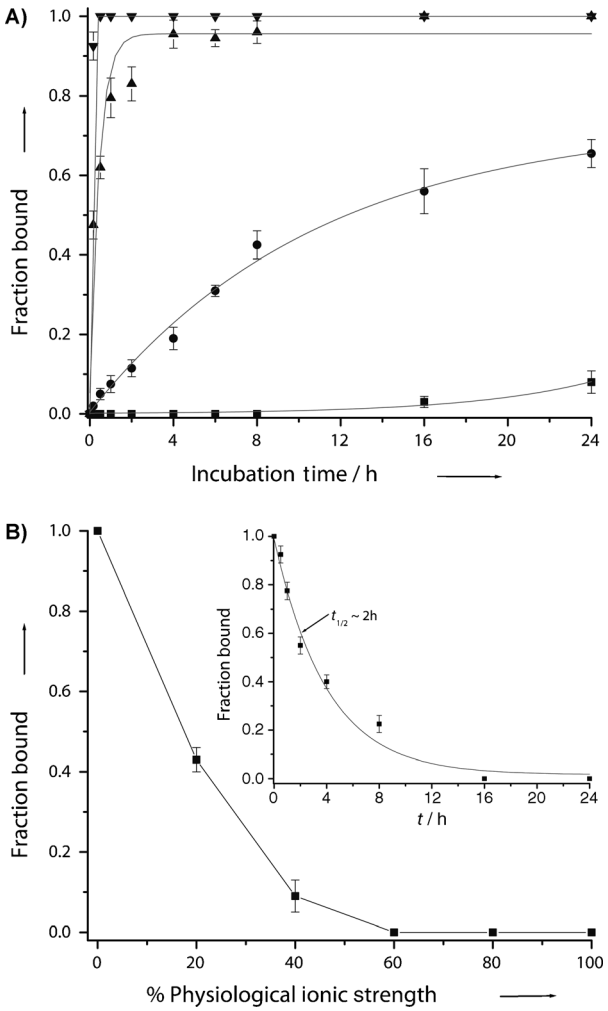

Figure 3. Effects of: A) temperature; 10 mM NaPi buffer was used at the indicated temperatures; ■, 4 °C, $t_{1/2}$ 195 h; ●, 22 °C, $t_{1/2}$ 16 h; ▲, 37 °C, $t_{1/2}$ 1 h; ▼, 50 °C, $t_{1/2}$ 20 h; and B) ionic strength on the invasion efficiency; different percentages of physiological ionic strength (2 mM $MgCl_2$, 150 mM KCl, 10 mM NaPi) were used at 37 °C for 16 h followed by electrophoretic separation and SYBR-Gold staining. The fraction bound was determined by gel-shift assays following incubation of 0.1 μM DNA containing **PM** binding site with 2 μM $^{(R)\text{-MP}}\gamma$PNA1. Inset: profile of the $^{(R)\text{-MP}}\gamma$PNA1–DNA complex dissociating as a function of time after reconstitution of the sample with 100% physiological ionic strength. The $t_{1/2}$ is defined as the time required to reach 50% binding.

$^{(R)\text{-MP}}\gamma$PNA8b or $^{(R)\text{-MP}}\gamma$PNA8bm) with DNA containing single-base mismatches (Figure S4 in the Supporting Information) resulted in formation of the invasion complex. However, it should be emphasized that as with any intermolecular recognition event, caution must be exercised in designing the nucleobase sequence to minimize self-hybridization because of the strong $^{(R)\text{-MP}}\gamma$PNA–$^{(R)\text{-MP}}\gamma$PNA interaction, in order to achieve optimum invasion.

In summary, we have shown that (*R*)-MiniPEG-containing γPNAs, 13 to 20 nucleotides in length, can invade any sequence of double helical B-DNA. Recognition, in this case, occurs in a highly sequence-specific manner (vide infra) through Watson–Crick base pairing. The crystal structure of a $^{(S)\text{-Me}}\gamma$PNA–DNA duplex has been determined,[44] and shows the methyl groups projecting outward toward the solvent. We do

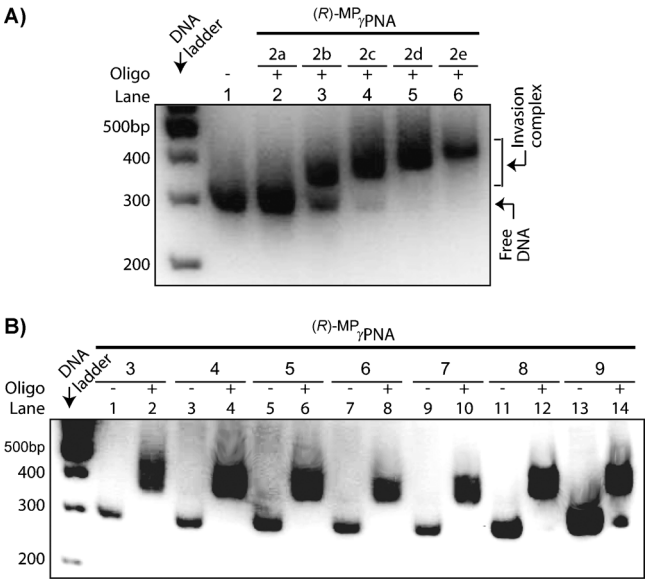

Figure 4. Results of gel-shift assays showing the effects of oligonucleotide: A) size, and B) sequence composition on the invasion efficiency. Gel-shift assays were performed under identical conditions as stated in the Figure 1 legend at a DNA/$^{(R)\text{-MP}}\gamma$PNA ratio of 20:1. The sequences of the $^{(R)\text{-MP}}\gamma$PNA oligonucleotides and corresponding DNA targets are shown in Table 1 and Table S1 in the Supporting Information, respectively.

not expect the MP side chains to behave any differently. The main advantages of MP over Me are improvements in water solubility and biocompatibility, and suppression of aggregation and nonspecific binding. Though $^{(R)\text{-MP}}\gamma$PNA binding is presently limited to relatively low ionic strengths, like all other strand invading PNAs with the exception of triplex binding, this is not because they are unable to gain access to the nucleobase targets under physiological conditions (ionic strengths and temperatures), but rather because they are unable to compete with the native complementary DNA strand. This is predominantly a thermodynamic, rather than a kinetic, issue that could be resolved through molecular design. We have already demonstrated certain aspects of this design through covalent attachment of DNA intercalating agents[37] and replacement of natural nucleobases with synthetic analogues.[41] The $^{(R)\text{-MP}}\gamma$PNAs are attractive, as compared to the other classes of antigene reagents that have been developed to date, because they are relatively easy to synthesize and they hybridize to their targets in a highly sequence specific manner in accordance with the simple rules of Watson–Crick base-pairing. Such antigene reagents could be employed in a number of biotechnology and genomic applications, including recombinant DNA,[27] genome mapping[45] and chromatin capture,[46] as well as in vivo, including gene regulation[28,47] and gene correction.[48]

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Keywords: DNA recognition · gamma-PNA · nucleic acids · strand invasion · Watson–Crick base pairing

[1] N. T. Thuong, C. Helene, *Angew. Chem.* **1993**, *105*, 697; *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 666.
[2] P. E. Nielsen, *Chem. Eur. J.* **1997**, *3*, 505.
[3] C. Denison, T. Kodadek, *Chem. Biol.* **1998**, *5*, R129.
[4] M. D. Frank-Kamenetskii, *Nature* **1987**, *328*, 17.
[5] H. E. Moser, P. B. Dervan, *Science* **1987**, *238*, 645.
[6] J. S. Sun, C. Helene, *Curr. Opin. Struct. Biol.* **1993**, *3*, 345.
[7] C. Giovannangeli, C. Helene, *Antisense Nucleic Acid Drug Dev.* **1997**, *7*, 413.
[8] C. L. Kielkopf, S. White, J. W. Szewczyk, J. M. Turner, E. E. Baird, P. B. Dervan, D. C. Rees, *Science* **1998**, *282*, 111.
[9] J. M. Gottesfeld, L. Neely, J. W. Trauger, E. E. Baird, P. B. Dervan, *Nature* **1997**, *387*, 202.
[10] S. White, J. W. Szewczyk, J. M. Turner, E. E. Baird, P. B. Dervan, *Nature* **1998**, *391*, 468.
[11] C. C. O'Hare, D. Mack, M. Tandon, S. K. Sharma, J. W. Lown, M. L. Kopka, R. E. Dickerson, J. A. Hartley, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 72.
[12] H. A. Greisman, C. O. Pabo, *Science* **1997**, *275*, 657.
[13] D. Jantz, B. T. Amann, G. J. Gatto, J. M. Berg, *Chem. Rev.* **2004**, *104*, 789.
[14] R. R. Beerli, C. F. I. Barbas, *Nat. Biotechnol.* **2002**, *20*, 135.
[15] M. Moore, A. Klug, Y. Choo, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 1437.
[16] C. W. Garvie, C. Wolberger, *Mol. Cell* **2001**, *8*, 937.
[17] D. M. Gowers, K. R. Fox, *Nucleic Acids Res.* **1999**, *27*, 1569.
[18] J. J. Kelly, E. E. Baird, P. B. Dervan, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 6981.
[19] C. L. Kielkopf, E. E. Baird, P. B. Dervan, D. C. Rees, *Nat. Struct. Biol.* **1998**, *5*, 104.
[20] P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, *Science* **1991**, *254*, 1497.
[21] P. E. Nielsen, *Acc. Chem. Res.* **1999**, *32*, 624.
[22] M. Egholm, P. E. Nielsen, O. Buchardt, R. H. Berg, *J. Am. Chem. Soc.* **1992**, *114*, 9677.
[23] P. E. Nielsen, L. Christensen, *J. Am. Chem. Soc.* **1996**, *118*, 2287.
[24] T. Bentin, P. E. Nielsen, *Biochemistry* **1996**, *35*, 8863.
[25] S. V. Smulevitch, C. G. Simmons, J. C. Norton, T. W. Wise, D. R. Corey, *Nat. Biotechnol.* **1996**, *14*, 1700.
[26] X. Zhang, T. Ishihara, D. R. Corey, *Nucleic Acids Res.* **2000**, *28*, 3332.
[27] M. Komiyama, Y. Aiba, Y. Yamamoto, J. Sumaoka, *Nat. Protoc.* **2008**, *3*, 655.
[28] B. A. Janowski, K. Kaihatsu, K. E. Huffman, J. C. Schwartz, R. Ram, D. Hardy, C. R. Mendelson, D. R. Corey, *Nat. Chem. Biol.* **2005**, *1*, 210.
[29] I. V. Smolina, V. V. Demidov, V. A. Soldatenkov, S. G. Chasovskikh, M. D. Frank-Kameneteskii, *Nucleic Acid Res.* **2005**, *33*, e146.
[30] T. Bentin, H. J. Larsen, P. E. Nielsen, *Biochemistry* **2003**, *42*, 13987.
[31] K. Kaihatsu, R. H. Shah, X. Zhao, D. R. Corey, *Biochemistry* **2003**, *42*, 13996.
[32] J. Lohse, O. Dahl, P. E. Nielsen, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11804.
[33] V. V. Demidov, E. Protozanova, K. I. Izvolsky, C. Price, P. E. Nielsen, M. D. Frank-Kamenetskii, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5953.
[34] G. He, S. Rapireddy, R. Bahal, B. Sahu, D. H. Ly, *J. Am. Chem. Soc.* **2009**, *131*, 12088.
[35] J. Milton Harris, R. B. Chess, *Nat. Rev. Drug Discovery* **2003**, *2*, 214.
[36] B. Sahu, I. Sacui, S. Rapireddy, K. J. Zanotti, R. Bahal, B. A. Armitage, D. H. Ly, *J. Org. Chem.* **2011**, *76*, 5614.
[37] S. Rapireddy, G. He, S. Roy, B. A. Armitage, D. H. Ly, *J. Am. Chem. Soc.* **2007**, *129*, 15596.
[38] P. M. Scholten, A. Nordheim, *Nucleic Acids Res.* **1986**, *14*, 3981.
[39] J. C. Furlong, D. M. J. Lilley, *Nucleic Acids Res.* **1986**, *14*, 3995.
[40] S. W. Santoro, G. F. Joyce, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4262.
[41] S. Rapireddy, R. Bahal, D. H. Ly, *Biochemistry* **2011**, *50*, 3913.
[42] Z. Liu, S. S. Venkatesh, C. C. Maley, *BMC Genomics* **2008**, *9*, 509.
[43] V. Chenna, S. Rapireddy, B. Sahu, C. Ausin, E. Pedroso, D. H. Ly, *ChemBioChem* **2008**, *9*, 2388.
[44] J. I. Yeh, B. Shivachev, S. Rapireddy, M. J. Crawford, R. R. Gil, S. Du, M. Madrid, D. H. Ly, *J. Am. Chem. Soc.* **2010**, *132*, 10717.
[45] J. Lin, Z. Lai, C. Aston, J. Jing, T. S. Anantharaman, B. Mishra, O. White, M. J. Daly, K. W. Minton, J. C. Venter, D. C. Schwartz, *Science* **1999**, *285*, 1558.
[46] J. Dekker, K. Rippe, M. Dekker, N. Kleckner, *Science* **2002**, *295*, 1306.
[47] N. E. Mollegaard, O. Buchardt, M. Egholm, P. E. Nielsen, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3892.
[48] K. M. Vasquez, L. Narayanan, P. M. Glazer, *Science* **2000**, *290*, 530.

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