# Specific versus Nonspecific Binding of Cationic PNAs to Duplex DNA

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ABSTRACT Although peptide nucleic acids (PNAs) are neutral by themselves, they are usually appended with positively charged lysine residues to increase their solubility and binding affinity for nucleic acid targets. Thus obtained cationic PNAs very effectively interact with the designated duplex DNA targets in a sequence-specific manner forming strand-invasion complexes. We report on the study of the nonspecific effects in the kinetics of formation of sequence-specific PNA-DNA complexes. We find that in a typical range of salt concentrations used when working with strand-invading PNAs (10–20 mM NaCl) the PNA binding rates essentially do not depend on the presence of nontarget DNA in the reaction mixture. However, at lower salt concentrations (<10 mM NaCl), the rates of PNA binding to DNA targets are significantly slowed down by the excess of unrelated DNA. This effect of nontarget DNA arises from depleting the concentration of free PNA capable of interacting with DNA target due to adhesion of positively charged PNA molecules on the negatively charged DNA duplex. As expected, the nonspecific electrostatic effects are more pronounced for more charged PNAs. We propose a simple model quantitatively describing all major features of the observed phenomenon. This understanding is important for design of and manipulation with the DNA-binding polycationic ligands in general and PNA-based drugs in particular.

## INTRODUCTION

Peptide nucleic acids (PNAs) are nucleic acid analogs composed of a neutral pseudopeptide/protein-like (rather than sugar-phosphate) backbone appended with regular or modified nucleobases (Nielsen et al., 1991). This clever design allows PNAs to retain the specificity of Watson-Crick (and Hoogsteen) recognition while increasing their binding affinity and biostability. Therefore PNAs constitute a unique class of potent DNA- (and RNA-) binding ligands capable of interacting with their target sites efficiently and in a highly sequence-specific manner (PNA chemistry and applications are reviewed by Dueholm and Nielsen, 1997; Uhlmann et al., 1998; Ray and Nordén, 2000; Nielsen, 2001). A remarkable feature of PNAs consists in their ability to sequence-specifically target not only single-stranded (ss) nucleic acids but also DNA in double-stranded (ds) form yielding very stable complexes.

Until recently, PNAs carrying only pyrimidine bases represented the most potent type of dsDNA targeting probes interacting with their targets via so-called triplex invasion mechanism. This involves formation of a hybrid triplex by two PNA strands and a homopurine strand of dsDNA whereas the other DNA strand is displaced from the double helix (Nielsen et al., 1993; Demidov et al., 1993, 1994, 1995). To increase the efficiency of this trimolecular reaction, two PNA oligomers participating in triplex formation are joined by a flexible linker resulting in bisPNA (Egholm et al., 1995; Griffith et al., 1995). A significant flaw of the original PNA design consisted in a poor solubility of PNAs. This drawback was overcome by incorporation of one or several lysine residues usually at the end of the PNA chain. In addition, this resulted in further improvement of PNA-DNA complex association kinetics without compromising specificity of sequence recognition (Kuhn et al., 1998 and 1999). Still, even lysine-labeled bisPNAs can invade only homopurine-homopyrimidine sites in dsDNA.

To relax these stringent requirements, modified bases have been introduced into PNA yielding pseudo-complementary PNAs (pcPNAs). The pcPNAs carry ordinary guanines and cytosines, whereas 2,6-diaminopurines (D) and 2-thiouracils ($^{s}$U) are substituted for adenines and thymines, respectively (Lohse et al., 1999; Izvolsky et al., 2000; Demidov et al., 2002). This modification impedes basepairing between two pcPNA strands, while enhancing the affinity to corresponding nonmodified sequences within dsDNA (Kutyavin et al., 1996). The notion of pseudo-complementarity stands for the ability of two corresponding pcPNA strands to hybridize to the complementary DNA sequences via Watson-Crick basepairing whereas the two strands have much lower affinity to each other. The consequence of this is yet another mechanism of dsDNA sequence recognition displayed by PNAs: the double-duplex invasion. As Fig. 1 schematically shows, a pair of pcPNAs pries dsDNA open via formation of two Watson-Crick type duplexes with the corresponding complementary stretches of mixed-sequence DNA.

Although hybridization of two pcPNA oligomers with each other is hampered by the presence of modified bases, the formation of weak pcPNA duplexes has been observed (Demidov et al., 2002; see this article, Fig. 1, *Reaction 2*). The formation of pcPNA duplexes affects the kinetics of the pcPNA binding to DNA, since only free, not paired pcPNAs, are capable of interacting with dsDNA targets. In addition, pcPNA self-association results in the formation of highly charged molecules. In fact, if a single pcPNA carries from

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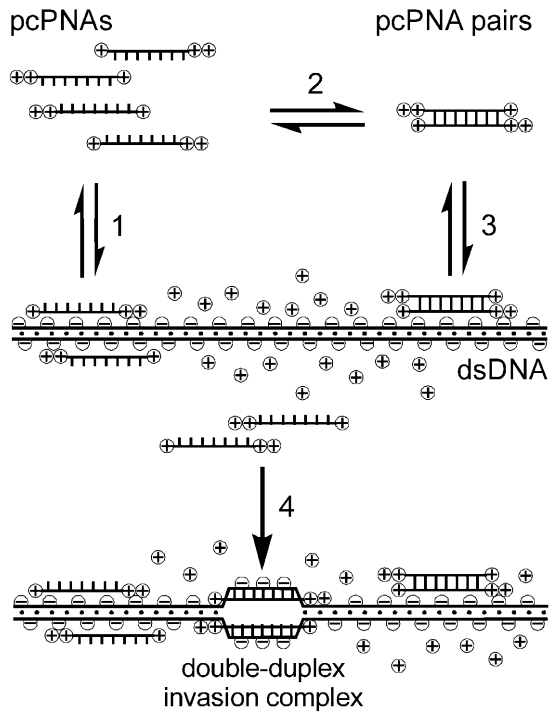


FIGURE 1 Proposed pathways for specific and nonspecific interaction of cationic pcPNAs with dsDNA. Reaction 1, reversible binding of pcPNAs to unrelated (nontarget) dsDNA; Reaction 2, formation of pcPNA duplexes via association of two matching pcPNAs; Reaction 3, reversible binding of highly charged pcPNA duplexes to DNA; and Reaction 4, irreversible binding of free unpaired pcPNAs with the dsDNA target site resulting in the formation of the sequence-specific double-duplex invasion complex. The pathway for bisPNA binding to dsDNA target site involves Reaction 1 followed by Reaction 4, since bisPNAs do not associate to form PNA pairs.

two to four positive charges, the pcPNA duplex will carry up to eight positive charges.

The ionic conditions play a very important role in interaction of PNAs with dsDNA. The strand-invasive PNA binding to complementary dsDNA sites is well known to be inhibited by increasing salt concentrations (Wittung et al., 1996; Bentin and Nielsen, 1996; Izvolsky et al., 2000). Accordingly, this effect may generate certain problems for the in vivo gene-targeting approaches since intracellular PNA targeting should inevitably occur at a physiological, relatively high salt concentration. Fortunately, the inhibitory salt effect can be alleviated in vivo by natural DNA supercoiling (Bentin and Nielsen, 1996; Larsen and Nielsen, 1996; Izvolsky et al., 2000). As to in vitro applications, most of them could be accomplished with PNA targeting at low salt. Moreover, such conditions are favorable in vitro not only because of faster PNA-DNA binding but also due to usually better stability of sequence-specific PNA-DNA strand-invasion complexes in low-salt solutions. Both in vitro and in vivo, use of cationic PNAs is advantageous in terms of binding kinetics (Kuhn et al., 1999).

PNAs are polycations, and can exhibit a strong nonspecific binding to DNA macroanions in addition to their specific binding to DNA target sites (Fig. 1). This nonspecific binding is similar to the behavior displayed by other cationic DNA-binding ligands such as cationic oligopeptides and polyamines (Braunlin et al., 1982; Rouzina and Bloomfield, 1996; Padmanabhan et al., 1997; Potaman and Sinden, 1995, 1998). The goal of the present work consists in studying the effect of nonspecific polycation-macroanion interaction on the kinetics of the specific complex formation between PNA and dsDNA.

We have found that, at low ionic strengths, the kinetics of association of cationic PNAs with their dsDNA targets is vastly affected by the presence of unrelated nontarget DNA. We have observed a >10-fold drop in the rate of PNA-DNA complex formation with the 15-fold increase in the concentration of nontarget DNA. We attribute the inhibitory effect of unrelated DNA to a nonspecific reversible association of positively charged PNA and/or PNA duplexes with DNA depleting the concentration of free PNA in the reaction mixture (Fig. 1). The corresponding interaction has a substantial electrostatic contribution; an enhanced inhibition is observed for PNAs carrying more positive charges. An increase in the salt concentration leads to more efficient screening of electrostatic attraction between PNA and DNA, thus relaxing the dependence of association kinetics on the concentration of nonspecific DNA. The observed phenomenon has implications for designing PNA-based probes for dsDNA and their applications both in vitro and in vivo.

## MATERIALS AND METHODS

### Materials

PNA oligomers used in the present study (summarized in Table 1) have been obtained following protocols described before (Egholm et al., 1995; Lohse et al., 1999). PNAs are written, using the peptide convention, from the N-terminus to the C-terminus. To increase their solubility and affinity to DNA targets, all PNAs are appended with lysines at their termini; thus at physiological pH, PNAs are positively charged. The number of charges carried by each PNA oligomer is indicated in Table 1. PNA concentrations were determined spectrophotometrically using the following nucleotide extinction coefficients at 260 nm: 11,700 (G), 10,200 ($^{S}$U), 7600 (D), 6600 (C), 8800 (T), and 3000 (J) $M^{-1}$ $cm^{-1}$.

Recombinant plasmids, p8, p10, and p10bis, carrying PNA-binding sites, are pUC19 derivatives with corresponding inserts cloned into BamHI (all restriction endonucleases used herein were purchased from New England Biolabs, Beverly, MA) site of the polylinker. Insert in p8 contains the binding site for self-pseudo-complementary 8-mer PNA *1690*, $^{5'}$TTGATCAA. Target sequence included in p10, $^{5'}$GCATGTTTGA, is designed for a pair of pcPNAs *1914* and one of *1915*, *1916*, or *1917*; the latter three have identical nucleotide sequence but differ in the number of terminal lysine residues, and consequently charge. Lastly, p10bis carries the homopurine target site for bisPNA *522*, $^{5'}$AAGAAGAAAA.

The plasmids were digested with PvuII restriction endonuclease yielding a 350-bp fragment, which carries the PNA target. Alternatively, a 265-bp fragment containing a PNA-binding site in the center was generated by PCR with *Taq* DNA polymerase (Promega Biosciences, San Luis Obispo, CA) using corresponding plasmid as a template. All DNA samples were purified by phenol extraction and subsequent gel filtration through Sephadex G-50 pre-equilibrated with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

**TABLE 1 PNA oligomers used in this study**

| PNA number | PNA sequence* | Charge† | Target plasmid |
|---|---|---|---|
| 1690 | H-Lys$_2$-$^s$U$^s$UGD$^s$UCDD-Lys-NH$_2$ | +4 | p8 |
| 1914 | H-Lys$_2$-$^s$UCDDDCD$^s$UGC-Lys-NH$_2$ | +4 | p10 |
| 1915 | H-GCD$^s$UG$^s$U$^s$U$^s$UGD-Lys-NH$_2$ | +2 | p10 |
| 1916 | H-Lys-GCD$^s$UG$^s$U$^s$U$^s$UGD-Lys-NH$_2$ | +3 | p10 |
| 1917 | H-Lys$_2$-GCD$^s$UG$^s$U$^s$U$^s$UGD-Lys-NH$_2$ | +4 | p10 |
| 522 | H-TTJTTJTTTT-(egl)$_3$-TTTTCTTCTT-Lys-NH$_2$ | +2 | p10bis |

*PNAs carry the following modified bases: *J*, pseudoisocytosine (Egholm et al., 1995); *D*, 2,6-diaminopurine; and $^sU$, 2-thiouracil (Lohse et al., 1999). *Lys* is a lysine residue; *egl* denotes linker unit given by 8-amino-3, 6-dioxaoctanoic acid.

†Total charge is given by protonated ε-amino groups of Lys residues and one extra amino group of N-terminal Lys (Demidov and Frank-Kamenetskii, 2001).

DNA concentration was assessed spectrophotometrically assuming 1 $OD_{260}$ corresponds to 75 $\mu$M of basepairs.

## Association kinetics measurements

Unless stated otherwise, binding kinetics experiments were carried out by combining target DNA (PvuII restriction digest of respective plasmid or a PCR fragment) and PNA stock in a 28-$\mu$L reaction volume and incubating the reaction mixture at 37°C. To vary the total concentration of nonspecific DNA, the reaction mixture was appended with appropriate amounts of linearized pUC19, which does not carry binding sites for our PNAs. Most of the kinetic experiments were conducted under low ionic strength conditions in 10 mM TE (pH 7.4) buffer which contains 2 mM $Na^+$. The 4-$\mu$L aliquots were removed from the reaction mixture at certain time intervals and the binding reaction was stopped by addition of NaCl to a final concentration of 200 mM. In most experiments, PNA concentration was maintained at 625 nM (of strands), which is at least 10 times higher than the concentration of the target sites for sequence-specific binding of PNAs to DNA. We varied total concentration of nonspecific DNA from 10 to 167 $\mu$M (of basepairs). For example, 10 $\mu$M of a 265-bp PCR fragment corresponds to 38 nM in PNA target sites, or 160 $\mu$M of p8/PvuII digest is 60 nM in PNA target sites. Note that the pH of our reaction solutions does not change upon addition of excess DNA.

The time course of PNA binding to the DNA target site was monitored by resolving the samples through 8% nondenaturing polyacrylamide gels with 1× TBE (90 mM Tris, 90 mM boric acid, 1 mM EDTA, pH 8.0) as a running buffer. DNA band patterns were visualized by ethidium bromide staining and detected by the charge-coupled device camera coupled with IS-1000 digital imaging software (Alpha Innotech, San Leandro, CA). The extent of PNA-DNA complex formation was determined by measuring the normalized intensities of the faster (free DNA fragment) and slower (PNA-DNA complex) migrating bands (Fig. 2 *A*). All kinetic measurements were collected as triplicates.

Pseudo-first-order rate constants of PNA-DNA association, $k_{ps}$, defined by the equation

$$C = 1 - \exp(-k_{ps}t) \quad (1)$$

(where $C$ is the fraction of PNA-DNA complexes formed at time $t$), were measured as the slope of $-\ln(1-C)$ versus time.

## Thermal denaturation experiments

Melting curves of pcPNA duplexes were collected by following the absorbance at 280 nm (PNA *1690*) or 272 nm (PNA *1914/1915* and *1914/1917*) using a Varian Cary 4G UV/visible spectrophotometer (Varian, Cary, NC). Temperature in the optical cell was gradually increased at the heating rate of ~0.8°C/min using a circulating water bath attached to the cell holder. Melting experiments were conducted in TE (pH 7.4) buffer at 4 $\mu$M of each strand for PNA pairs *1914/1915* and *1914/1917* and at 8 $\mu$M of strands for PNA *1690*. Melting profiles were normalized as described previously by Marky and Breslauer (1987).

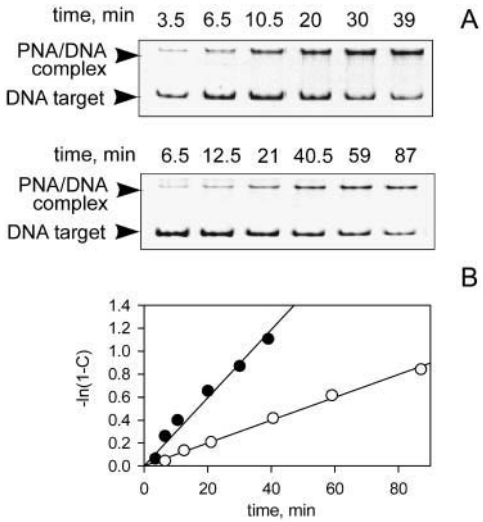


FIGURE 2 PNA-DNA association obeys pseudo-first-order kinetics but it is affected by the total concentration of nontarget DNA present in the reaction mixture. (*A*) Gel-electrophoresis shift assay is used to monitor time course of pcPNAs *1690* binding to DNA target site located within a 350-bp fragment of p8/PvuII digest. Both experiments were performed at 37°C in 10 mM TE (pH 7.4), which contains 2 mM $Na^+$. The concentration of pcPNAs and DNA target sites was 625 nM and 23 nM, respectively. The amount of nonspecific DNA (in bp) in the reaction mixture was varied from 60 $\mu$M (*upper panel*) to 131 $\mu$M (*lower panel*) by addition of appropriate amounts of linearized pUC19. (*B*) The yield of the PNA/DNA complex, *C*, fits linear regression; total concentrations of nonspecific DNA (in bp): 60 $\mu$M (●) and 131 $\mu$M (○). Pseudo-first-order rate constants given by the slopes of linear regression are 0.0297 and 0.0100 $min^{-1}$, respectively.

# RESULTS

## At low salt, nonspecific DNA affects kinetics of PNA binding to dsDNA

Using gel electrophoresis mobility shift assay we attain the time-course of the pcPNA *1690* binding to a DNA target site located within a 350-bp fragment of the p8/PvuII digest (Fig. 2 *A*). Formation of the specific double-duplex complex by a pair of pcPNAs with the DNA target results in a distinct retardation of the DNA fragment when resolved by gel electrophoresis under native conditions (Lohse et al., 1999; Izvolsky et al., 2000). In the two experiments shown we kept the target concentration constant at 23 nM and varied the concentration of unrelated DNA by the addition of a linearized pUC19, which does not carry any target sites for our pcPNAs. An increase of the total concentration of nonspecific DNA in the reaction mixture from 60 to 131 $\mu$M of basepairs results in an appreciable inhibition of the

PNA-DNA complex formation. Linearity of the plots in Fig. 2 *B* verifies the applicability of the pseudo-first-order kinetics for our experimental conditions.

We obtained the kinetic parameters for the double-duplex pcPNA-DNA complex formation over a wide range of total DNA concentrations at low salt. Fig. 3 presents the association pseudo-first-order rate constants for the PNA *1690* binding to dsDNA target. We used three methods to vary the concentration of unrelated DNA. One set of experiments involved increasing the p8/PvuII restriction digest concentration from 20 to 160 $\mu$M of basepairs (the PNA target is located within the 350-bp fragment; however, there is also a large ~2300-bp plasmid fragment). Note, that in this case, the concentration of the PNA target site also varied from 7.5 to 60 nM, respectively. Further increase in the DNA concentration was achieved by addition of a linearized pUC19 to p8/PvuII at 23 nM of target sites. For the other extreme case of very low DNA concentrations we worked with the PCR-amplified, 265-bp-long DNA fragment carrying the PNA binding site. Kinetic measurements for three types of DNA samples shown in Fig. 3 follow the same tendency: $k_{ps}$ gradually decreases with increasing concentration of unrelated DNA.

To ensure that the inhibitory effect of nonspecific DNA on the PNA-DNA association is not an intrinsic feature of pcPNAs only we assessed the concentration-dependence of the binding kinetics for another type of cationic PNA—i.e., bisPNA. Fig. 3 presents the corresponding rate constants. The data display the same (though less pronounced) tendency of the rate constant decrease with increasing total concentration of unrelated DNA. This proves that the observed phenomenon of slowing kinetics with increasing DNA concentration is a general characteristic of the cationic PNA interaction with DNA.

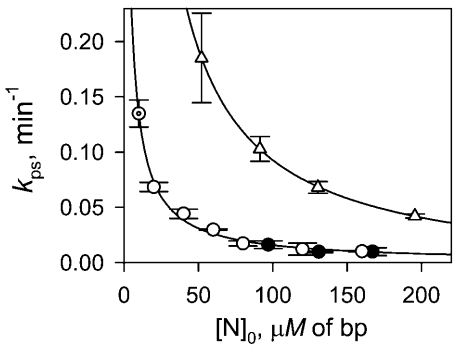


FIGURE 3 Dependence of $k_{ps}$ on the total concentration of nontarget DNA, $[N]_0$. Pseudo-first-order rate constants for pcPNA *1690* (*circles*) and bisPNA *522* (*triangles*) binding to corresponding DNA targets. Concentration of PNA was 625 nM in both cases. DNA concentration was varied by changing the amounts of the PCR-amplified, 265-bp fragment (⊙), PvuII restriction digest of corresponding plasmid (○ and △), and addition of nontarget linearized pUC19 (●). Experiments were performed in 10 mM TE, pH 7.4 (pcPNA) and pH 7.0 (bisPNA) buffer at 37°C. The lines are drawn to guide the eye.

## Charge dependence of the pcPNA-dsDNA binding kinetics

To explore the origin of the nontarget DNA inhibition we studied the effect of the charge at pcPNAs termini on the pcPNA-DNA complex formation. The pcPNA pairs used in this set of experiments have identical sequences; they differ in the number of lysine residues attached to their termini resulting in the following total charges per pcPNA pair: PNA *1914/1915* pair carries six positive charges, PNA *1914/1916* pair carries seven charges, and PNA *1914/1917* pair carries eight charges. At low salt the kinetic analysis for all three PNA pairs displays the same tendency toward slower binding rates with increasing concentration of nontarget DNA (Fig. 4). There is a distinct dependence of binding kinetics on the number of charges carried by a PNA pair. At low DNA concentration, pseudo-first-order kinetic constant drops from 0.086 min$^{-1}$ for PNA *1914/1915* to 0.020 min$^{-1}$ for PNA *1914/1917*, which corresponds to the addition of two positive charges. The difference in the association kinetics is less pronounced at higher DNA concentrations. The effect of charges carried by PNAs reveals a substantial electrostatic contribution into nonspecific interaction of PNA with DNA.

## Effect of ionic strength on binding kinetics

It has been established before that due to the strand invasive mode of PNA interaction with dsDNA targets an increased salt concentration slows down the PNA binding by stabilizing dsDNA (Wittung et al., 1996; Bentin and Nielsen, 1996; Izvolsky et al., 2000). Electrostatic screening of the Coulomb attraction between positively charged PNA and

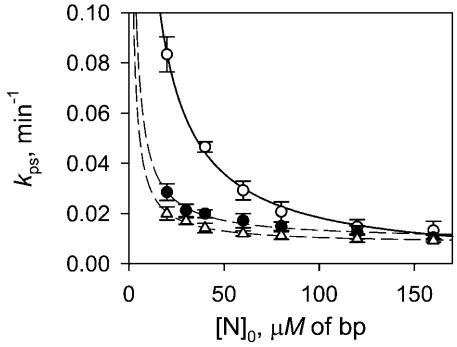


FIGURE 4 The effect of the total charge carried by PNAs on the pcPNA-DNA association kinetics. PNA pairs *1914/1915* (○), *1914/1916* (●), and *1914/1917* (△) target identical DNA sequences located within a 350-bp fragment of p10/PvuII, but differ in the number of lysine residues (and consequently charges) attached to their termini (see Table 1). Kinetic experiments were carried out in 10 mM TE, pH 7.4, at 37°C. The PNA concentration was maintained constant at 625 nM (each strand), whereas $[N]_0$ varied by changing the concentration of the p10/PvuII restriction digest. The solid line presents the nonlinear fit of the kinetic data for PNA pair *1914/1915* according to Eq. A15; regression parameters are $K_2 = 0.4 \pm 0.2$ $\mu M^{-1}$ and $k_0[P]_0/2K_d = 0.76 \pm 0.3\,min^{-1}$. Broken lines are drawn to guide the eye.

negatively charged dsDNA also affects the PNA-DNA complex formation. If the former effect interferes mostly with the formation of the specific PNA-DNA complex, the later is also important for the nonspecific binding of PNA to negatively charged DNA. Varying the salt concentration will affect considerably electrostatic screening of the cationic shell around DNA, thus changing the nonspecific binding of PNA to DNA.

To assess the salt-dependence of the pseudo-first-order rate constants for pcPNAs binding to their targets at different amounts of total DNA, we measured the extent of PNA-DNA complex formation after 40-min incubation at 37°C. According to Eq. 1, for a given time of incubation, $k_{ps}$ is linearly proportional to $-\ln(1-C)$. Fig. 5 presents the dependence of $-\ln(1-C)$ on the ionic strength for PNA *1960* interaction with its target site. The concentration of DNA target was kept constant for these experiments; we varied the concentration of nonspecific DNA by addition of a linearized pUC19. Both samples display the expected tendency of slower binding kinetics with increasing ionic strength. The extent of PNA-DNA complex formation is almost identical at higher ionic strengths for the two concentrations of nontarget DNA. However, significant differences in the reaction efficiencies are observed when the sodium content drops below 10 mM. At low salt, binding of PNA *1690* to DNA target in the presence of 45 $\mu$M of nontarget DNA is almost two times faster when compared with the binding upon a twofold increase in the nontarget DNA concentration. Similar results were obtained for PNA pair *1914/1915* (data not shown). In fact, the observed difference in the binding kinetics for two samples at low salt is proportional to the amount of nonspecific DNA present in two samples (see Figs. 3 and 4).

It is important to emphasize that the effect of nontarget DNA on PNA binding to dsDNA target displays two distinct regimes depending on the ionic strength (Fig. 5). At sodium concentrations of 20 mM and higher, the presence of different amounts of nontarget DNA essentially does not affect the association kinetics of specific PNA-DNA complex. When sodium concentration drops below 10 mM, kinetics of specific binding strongly depends on the presence of unrelated DNA.

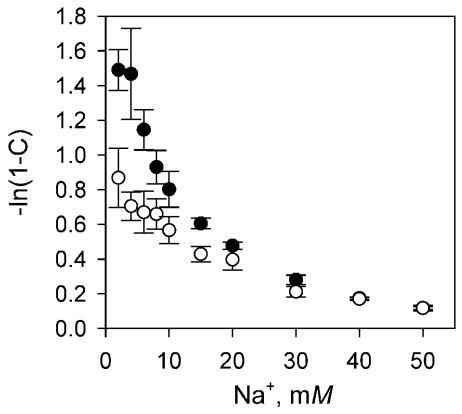


FIGURE 5 Salt-dependence of the pcPNA *1690* binding to DNA target sites. The extent of complex formation was assessed after 40-min incubation at 37°C in 10 mM TE, pH 7.4 buffer with addition of desirable amounts of NaCl. PNA concentration (571 nM) and DNA target concentration (17 nM) were kept constant. Total concentration of DNA (in bp) changed from 45 $\mu$M (●) to 90 $\mu$M (○) by addition of linearized pUC19.

## Effect of pcPNA duplex formation on PNA-DNA association kinetics

We have previously shown that two pseudo-complementary PNA strands are prone to association leading to formation of pcPNA duplexes (Demidov et al., 2002). This effect leads to depletion of free PNA concentration, thus affecting kinetics of specific PNA-DNA complex formation. To test whether duplex formation is still a factor at the low ionic strengths we use in the current study, we conducted thermal denaturation experiments (Fig. 6 *A*). Normalized melting profiles collected for the PNA *1914/1917* pair and PNA *1690* in TE (pH 7.4) buffer are virtually indistinguishable from the profiles we obtained earlier in TE (pH 7.6) buffer appended with 10 mM NaCl (Demidov et al., 2002). Also shown in Fig. 6 *A* is

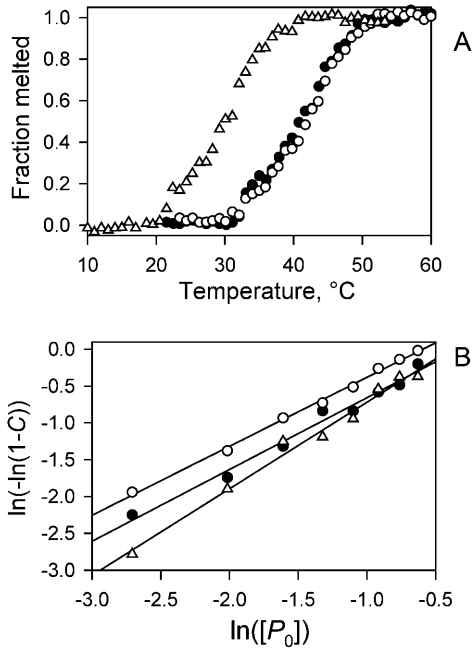


FIGURE 6 Effect of pcPNA duplex formation on the kinetics of PNA-DNA complex association. (*A*) Normalized thermal denaturation profiles for PNA pairs *1914/1915* (●), *1914/1917* (○), and PNA *1690* (△). (*B*) Double-logarithmic plots of $-\ln(1-C)$ after a 40-min (PNA pair *1914/1915* and PNA *1690*) or 70-min (PNA pair *1914/1917*) incubation of a target plasmid (p10/PvuII or p8/PvuII) in the presence of varying amounts of corresponding pcPNAs ($[P]_0$ increased from 67 nM to 533 nM). Straight lines present linear fits with slopes of 0.97, 0.94, and 1.17 for PNA pairs *1914/1915*, *1914/1917*, and PNA *1690*, respectively. Incubations were performed in 10 mM TE pH 7.4 buffer at 37°C. Concentration of nontarget DNA was 60 $\mu$M of bp.

the melting profile for the PNA *1914/1915* pair, which differs from the PNA *1914/1917* pair only in the number of terminal lysine residues and, consequently, in the total charge. Both PNA pairs undergo similar thermal melting transitions with the melting temperature of 42°C. Duplexes formed by self-complementary PNA *1690* are less stable with melting temperature of 30°C. Consequently, a substantial fraction (~70% for the 10-mer and ~10% for the 8-mer) of pcPNAs in our experiments remain in a duplex conformation at 37°C.

We allow for the pcPNA self-association when considering the overall process of PNA binding to dsDNA, including both sequence-specific and nonspecific interactions (see Appendix). When a substantial fraction of pcPNA exists in a duplex form (i.e., when PNA duplex melting temperature is above the reaction temperature) the pseudo-first-order rate constant is expected to increase linearly with the total concentration of pcPNA (Eq. A15). However, when the reaction conditions prevent the pcPNA self-association (i.e., when PNA duplex melting temperature is well below the reaction temperature), we expect a quadratic dependence on $[P]_0$ (Eq. A16).

Adopting the approach applied previously to study concentration dependence of PNA-DNA complex formation (Demidov et al., 2002), we assume that

$$k_{ps} \sim [P]_0^{\gamma}. \quad (2)$$

The exponent $\gamma$ can be estimated from the slope of $\ln(-\ln(1-C))$ plotted versus $\ln([P]_0)$. Fig. 6 *B* presents double-logarithmic plots for the PNA pairs *1914/1915*, *1914/1917*, and PNA *1690* obtained in the presence of 60 $\mu$M of unrelated DNA. Both sets of 10-mer pcPNAs follow a similar dependence with $\gamma$ close to 1. This result is consistent with the pcPNAs' melting profiles, indicating that, at our reaction temperature of 37°C, most of the pcPNAs are in the double-stranded form. In this case the dependence of $k_{ps}$ on the amount of unrelated DNA follows the expression given by Eq. A15.

Only slightly higher $\gamma$ of 1.17 is observed for PNA *1690*, indicating that although a small fraction of PNAs are in the double-stranded form, both pathways (i.e., adhesion of PNA oligomers and PNA duplexes to nontarget DNA) contribute to depletion of free PNA concentration affecting the kinetics of the specific complex formation.

## DISCUSSION

In our experiments we deal with a system consisting of polycations (PNA) and macroanions (DNA), as well as univalent counterions (see schematics in Fig. 1). According to the contemporary theory of polycation/macroanion systems (reviewed by Grosberg et al., 2002), their behavior differs dramatically depending on whether the total positive charge of all polycations in the solution (in our case, PNA molecules) is larger or lower than the total negative charge of all macroanions (in our case, DNA molecules). Our conditions always correspond to a strong excess (4–130 times) of the total DNA charge over the total PNA charge. This means that no charge inversion or the other effects, which are described by Nguyen and Shklovskii (2001) and reviewed by Grosberg et al. (2002), happen. These effects would occur in the opposite situation when the total PNA charge exceeds the total DNA charge. Under our conditions, the fact that we deal with the polycation (and a very strongly charged one) does not lead to any qualitative differences in the behavior of our system as compared to the singly charged cation. Thus, we can analyze our data keeping in mind a traditional picture of a macroanion surrounded by a cloud of cations (reviewed by Frank-Kamenetskii et al., 1987; Anderson and Record, 1990).

Figs. 3 and 4 clearly demonstrate that at low salt the kinetics of the PNA-DNA complex formation is sensitive to the total concentration of DNA present in the reaction mixture. Pseudo-first-order rate constants measured for binding of pcPNA substantially decrease with increasing concentration of unrelated nontarget DNA. We attribute this effect to nonspecific interactions of positively charged pcPNA with the negatively charged DNA. This nonspecific binding of PNA to nontarget DNA withdraws PNAs from the solution decreasing the effective concentration of PNA molecules capable of interacting with the specific DNA target. This process, shown schematically in Fig. 1, can occur by two different pathways: positively charged PNA oligomers can adhere to DNA matrix as single strands or following the formation of pcPNA duplexes. Note that the latter species will have significantly higher affinity to negatively charged DNA due to larger total charge carried by the duplex compared to PNA single strands.

Using a nonlinear fit according to Eq. A15 of our kinetic data for PNA *1914/1915* invasion presented in Fig. 4, we estimate the equilibrium constant $K_2$ of nonspecific binding for a PNA pair carrying six positive charges to be on the order of $10^5$ $M^{-1}$ (of basepairs) or $10^6$ $M^{-1}$ (per apparent binding site of ~10 bp). Similar values for equilibrium constants have been reported for nonspecific binding of cationic oligopeptides to DNA (Lohman et al., 1980; Mascotti and Lohman, 1993; Padmanabhan et al., 1997).

A substantial electrostatic component in the nonspecific PNA-DNA interaction manifests itself in the salt-dependence of the association kinetics presented in Fig. 5. As expected, for any complex formed via the strand-invasion mechanism, an increase in ionic strength slows down the kinetics of pcPNAs binding to double-stranded DNA targets (Izvolsky et al., 2000; Demidov et al., 2002). Elevated salt concentration also contributes to screening the Coulomb electrostatic attraction between positively charged PNAs and DNA, thus decreasing the nonspecific binding constant of PNA for DNA. According to Eqs. A15 and A16, when $K_1$ and $K_2$ are sufficiently small, the pseudo-first-order rate

constant becomes independent of the concentration of nonspecific DNA. This is consistent with the observed PNA-DNA association kinetics at [NaCl] >20 mM, where the rate constants are almost identical for the two concentrations of nonspecific DNA (Fig. 5). However, lowering salt concentration (and consequently increasing nonspecific binding constant) leads to a marked difference in the binding kinetics for the two samples. In fact, in agreement with Eq. A15, the PNA binding proceeds almost two times faster for the sample at $[N]_0 = 45\ \mu$M than for that at $[N]_0 = 90\ \mu$M. To summarize, PNA-DNA association kinetics displays two distinct regimes depending on the ionic strength: high salt (when screening of electrostatic attraction is efficient) and low salt (when inefficient screening leads to depletion of free PNA concentration thus affecting the kinetics of PNA binding to specific sites on DNA).

The existence of a weak nonspecific association of positively charged PNA with dsDNA has been implicated as a first step in PNA $T_{8-10}$ binding to poly(dA):poly(dT) (Wittung et al., 1996). The authors attribute this initial association to nonspecific PNA-DNA interactions that are mostly hydrophobic in nature and/or to transient random Hoogsteen-like basepairing with dsDNA polymer. However, these weak aggregates were not detected at elevated salt concentrations. This behavior is consistent with the nonspecific PNA-DNA association having a substantial electrostatic component, which we clearly observe in our experiments. Note that more efficient reversible adsorption of positively charged PNAs on unrelated ssDNA has also been reported by Tackett et al. (2002).

We attribute the inhibitory effect of unrelated DNA on the kinetics of specific targeting of DNA to nonspecific transient association of cationic PNA oligomers and pcPNA duplexes with negatively charged DNA lattice (Fig. 1). We demonstrate that the equilibrium between PNA bound nonspecifically to DNA and PNA in bulk solution is affected by salt concentration and depends on the total charge of PNA. These characteristics are consistent with a substantial electrostatic component of the nonspecific PNA-DNA association. At very low salt, when the screening of PNA-DNA electrostatic attraction is inefficient, we observe distinct dependence of kinetics on the presence of unrelated DNA. An increase in the ionic strength results in a drop of the nonspecific affinity of PNA to DNA to the point that its effect becomes negligible. In our experiments, the change in the PNA-DNA association kinetics with varying unrelated DNA content becomes insignificant at concentrations of sodium of 20 mM and higher. This result is in agreement with the large body of experimental data reporting both equilibrium and kinetic parameters for cationic PNA binding to DNA targets (Bentin and Nielsen, 1996; Wittung et al., 1997; Kuhn et al., 1998, 1999; Lohse et al., 1999; Demidov et al., 2002). Experimental conditions typically used for the PNA-DNA hybridization studies involve >10 mM $Na^+$, hence no significant adsorption of PNA to unrelated DNA is expected. Nevertheless, one must keep in mind that increasing the total charge carried by PNAs may noticeably enhance the nonspecific affinity of PNA toward DNA, thus affecting the overall kinetics and energetics of PNA-DNA interactions.

## CONCLUDING REMARKS

We have identified a nonspecific electrostatic binding of cationic lysine-tagged PNAs to nontarget dsDNA. Within the typically used range of salt concentrations of 20 mM and higher, this nonspecific PNA-DNA interaction does not affect the specific binding. Lower salt is well known to be preferable for PNA strand invasion as rates of specific PNA-DNA complex formation increase with decreasing salt (Fig. 5). However, decreasing the ionic strength below 10 mM NaCl results in pronounced adsorption of PNA on DNA that slows down the specific dsDNA targeting by depleting the concentration of free PNA.

Incorporation of positively charged residues into PNA is known to result in increased aqueous solubility of PNAs and in their pronounced efficiency of binding to dsDNA targets (Griffith et al., 1995; Bentin and Nielsen, 1996; Kuhn et al., 1998). Attaching longer cationic peptides has been used to improve affinity of sequence-specific binding of oligonucleotides and PNAs to their dsDNA targets (Smulevitch et al., 1996; Ishihara and Corey, 1999; Zhang et al., 2000; Kaihatsu et al., 2002). Our data show that at very low salt an increase in the total charge of PNA also enhances nonspecific adsorption of cationic PNA on negatively charged DNA. It is significant for future developments of PNA-based probes to optimize the net charge of PNAs keeping in mind the opposite trends of PNA binding to dsDNA with increasing charge: more efficient binding to the specific site on dsDNA and undesirable nonspecific adsorption of highly charged PNAs on DNA.

## APPENDIX

Formation of PNA-DNA strand invasion complexes in the presence of nontarget DNA, shown schematically in Fig. 1, can be described by the set of equations

$$P + N \overset{K_1}{\leftrightarrow} PN \tag{A1}$$

$$2P \overset{K_d}{\leftrightarrow} P_2 \tag{A2}$$

$$P_2 + N \overset{K_2}{\leftrightarrow} P_2N \tag{A3}$$

$$2P + D \overset{k_0}{\rightarrow} P_2D, \tag{A4}$$

where $D$ is target DNA, $N$ is nontarget DNA, and $P$ is pcPNA, which can hybridize with its pseudo-complementary counterpart resulting in the $P_2$ duplex. $P$ and $P_2$ interact nonspecifically with $N$ resulting in $PN$ and $P_2N$

complexes; $K_1$, $K_d$, $K_2$, and $k_0$ are corresponding equilibrium and rate constants for these processes. We assume that only free PNA can invade target site $D$, leading to formation of a sequence-specific complex $P_2D$.

We consider binding of PNAs to their DNA target as essentially irreversible, hence the PNA-DNA complex formation is fully described by the association rate constant $k_o$. This assumption has been validated by previous observations that typical PNA-DNA complexes are very stable and one can ignore their dissociation under our experimental conditions of neutral pH and low temperature (Cherny et al., 1993; Izvolsky et al., 2000; Demidov et al., 2002). Thus, the formation of a stable sequence-specific complex follows the kinetic equation

$$\frac{d[D]}{dt} = -k_0[P]^2[D]. \quad \text{(A5)}$$

Given that the processes described by Eqs. A1–A3 are fast compared to the formation of PNA-DNA complex (Eq. A4), we also assume that there is an equilibrium between free PNA, PNA duplexes, and PNA molecules reversibly bound to nonspecific DNA leading to a steady-state concentration of free PNA. Equilibrium constants $K_d$, $K_1$, and $K_2$ are defined by

$$K_d = \frac{[P_2]}{[P]^2} \quad \text{(A6)}$$

$$K_1 = \frac{[PN]}{[P][N]} \quad \text{(A7)}$$

$$K_2 = \frac{[P_2N]}{[P_2][N]}. \quad \text{(A8)}$$

In our experiments PNA has been used in a considerable excess over the DNA target, i.e., $[P]_0 \gg [D]_0$. Therefore, the free PNA concentration can be approximated by the equation

$$[P] \approx [P]_0 - ([PN] + 2[P_2] + 2[P_2N]). \quad \text{(A9)}$$

Since the steady-state concentration of free PNA given by Eq. A9 is constant over the course of the reaction, the pseudo-first-order formalism can be used to describe the kinetics of PNA interaction with its target DNA site. The pseudo-first-order rate constant for the PNA-DNA complex association is given by

$$k_{ps} = k_0[P]^2. \quad \text{(A10)}$$

The presence of nonspecific DNA as well as the PNA duplex formation results in depleting the effective PNA concentration, thus affecting kinetics of complex formation.

Combining Eqs. A6–A9 and noting that the concentration of nonspecific DNA remains constant during the course of the reaction (since in our experiments $[N]_0 \gg [P]_0$), we obtain the quadratic equation

$$\left(\frac{[P]}{[P]_0}\right)^2 + \left(\frac{[P]}{[P]_0}\right) \times \frac{1 + K_1[N]_0}{2K_d[P]_0(1 + K_2[N]_0)} - \frac{[P]_0}{2K_d[P]_0(1 + K_2[N]_0)} = 0. \quad \text{(A11)}$$

Equation A11 yields an explicit expression for the free PNA concentration as

$$[P] = \frac{[P]_0(1 + K_1[N]_0)}{4K_d(1 + K_2[N]_0)} \times \left(\sqrt{1 + \frac{8[P]_0K_d(1 + K_2[N]_0)}{(1 + K_1[N]_0)^2}} - 1\right). \quad \text{(A12)}$$

Let us consider two limiting cases with respect to the thermal stability of pcPNA duplexes: *case 1*, melting temperature of pcPNA duplexes is above the reaction temperature ($[P]_0K_d > 1$) and *case 2*, melting temperature is well below the reaction temperature ($[P]_0K_d \ll 1$).

In the first case the equilibrium in Reaction 2 (Fig. 1) is shifted toward the formation of PNA duplexes; these highly charged duplexes in turn interact nonspecifically with nontarget DNA. Since in this case $[P]_0K_d > 1$, Eq. A12 can be reduced to

$$[P] = \frac{\sqrt{[P]_0}}{\sqrt{2K_d(1 + K_2[N]_0)}}. \quad \text{(A13)}$$

Note that, since the numerical coefficient at $[P]_0K_d$ term in Eq. A12 is large, the approximation given by A13 is still valid for less stable PNA duplexes with melting temperatures close to the reaction temperature.

In the other extreme case of very weak pcPNA duplexes (*case 2* above, when $[P]_0K_d \ll 1$), the free PNA concentration is given by

$$[P] = \frac{[P]_0}{(1 + K_1[N]_0)}. \quad \text{(A14)}$$

Substituting Eqs. A13 or A14 to Eq. A10 we obtain the expression for the pseudo-first-order rate constant in the case of stable pcPNA duplexes as

$$k_{ps} = \frac{k_0[P]_0}{2K_d(1 + K_2[N]_0)}, \quad \text{(A15)}$$

and in the case of weak pcPNA duplexes,

$$k_{ps} = \frac{k_0[P]_0^2}{(1 + K_1[N]_0)^2}. \quad \text{(A16)}$$

Note that in our consideration we could neglect possible negative cooperativity effects, which might arise from the fact that the PNA oligomer or the PNA duplex occupies several basepairs on DNA while interacting nonspecifically with the DNA lattice (Eqs. A7 and A8; also see McGhee and von Hippel, 1974). Indeed, even in the case of the highest concentration of PNA duplexes (<70% of 625 nM for PNA *1914/1915*) and the lowest concentration of nonspecific DNA of 20 $\mu$M (and $K_2$ on the order of $10^5$ $M^{-1}$), we estimate a binding density of <15% of the lattice saturation for the 10-bp binding site.

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