Zn²⁺-Dependent Peptide Nucleic Acids Probes

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Peptide nucleic acid (PNA) is a mimic of DNA which has excellent binding affinity and sequence specificity toward oligo-nucleotides. Therefore, PNA has a strong potential as an antisense and antigene agent as it was established in cell-free systems[1] and in vivo.[2] In future therapeutical applications, it might be of advantage if PNA can be activated only in specific cells, thus avoiding the risk of adverse side effects in nontargeted cells. One of the approaches for this involves targeted delivery of PNA to specific cells by tethering PNA to the ligands recognized by cell receptors.[3] Another is the activation of PNA by the component present in the cell. Herein we show for the first time that Zn²⁺ can substantially increase binding affinity of terminally modified PNA to DNA targets in vitro. Zn²⁺ is present at high concentrations in brain,[4] pancreas,[5] and spermatozoa.[6] In breast cancer, tissues zinc levels are increased by 72%.[7] Ba²⁺/Sr²⁺- and Cu²⁺-dependent sequence-specific synthetic binders of DNA duplex as well as Gd³⁺-dependent binders of single-stranded DNA have been reported.[8,9a]

We have synthesized PNA in which a Zn²⁺ chelating ligand is conjugated via an aromatic linker. Intercalation of the aromatic linker is regulated by the metal coordination to the ligand (Figure 1). It has been recently shown that unsymmetrically modified naphthalene diimide (NADI) intercalates within PNA:DNA duplex and strongly stabilizes it.[10] Therefore, this aromatic fragment was chosen as a linker between PNA and the ligand (Figures 1 and 2). As ligands we have selected strong Zn²⁺ binders: bis(picolyl)amine (L1) and 1,4,8,11-tetraazacyclotetradecane (L2).

Syntheses of 2−4 have been described elsewhere.[9b,10] 1 and 1a were synthesized analogously to 3.[11] Binding of Zn²⁺ to 1 and 3 at equimolar concentrations (2 μM) is quantitative, since in these solutions no metal ion is detectable using the Zn²⁺-specific indicator Newport Green.[11,12] In contrast to melting transitions of DNA duplexes, those of PNA:DNA duplexes are typically quite broad.[11] After standard smoothing of melting curves and calculation of their first derivatives, one can observe usually one broad maximum rather than two maxima for transitions, T_m's of which differ by <10 °C. As is evident from measurements of averaged melting points of 1:6 duplex at different concentrations of Zn²⁺ (Figure 3), complex stoichiometry is not altered by PNA association with DNA. The melting temperature of the duplex of L1-NADI-gly-PNA (1) and 6 is strongly reduced in comparison with that of the duplex of NADI-gly-PNA (4) and 6 (ΔT_m = −7.6 °C, entries 1 and 4 in Table 1) and is practically identical to that of nonmodified 5:6 (entry 5, Table 1). Therefore, 1:6 should have a structure similar to that of nonmodified 5:6, with the L1-NADI residue freely rotating in solution. This suggestion is confirmed by the fact that the extinction of the NADI bands in UV−visible absorption spectrum of 1 is not affected by complementary 6. In contrast, the extinction of the corresponding bands in 4:6, where NADI is intercalating within the duplex, is reduced in comparison with that in the unbound PNA.[10b] Upon addition of Zn²⁺ to 1:6, the intensity of the NADI absorption bands is decreased,[11] indicating the formation of the structure of either intercalated (Figure 1B) or stacked at the end of
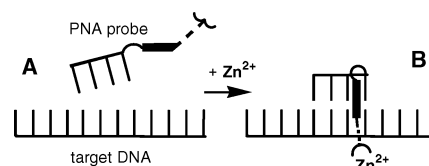

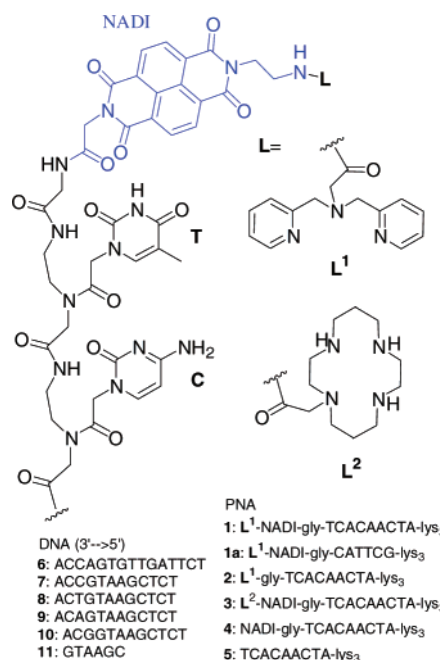**Figure 1.** Zn²-dependent binding of PNA probes to target DNA. Intercalator is shown as a thick line.

DNA (3'-->5')
6: ACCAGTGTTGATTCT
7: ACCGTAAGCTCT
8: ACTGTAAGCTCT
9: ACAGTAAGCTCT
10: ACGGTAAGCTCT
11: GTAAGC

PNA
1: L1-NADI-gly-TCACAACTA-lys₃
1a: L1-NADI-gly-CATTCG-lys₃
2: L1-gly-TCACAACTA-lys₃
3: L2-NADI-gly-TCACAACTA-lys₃
4: NADI-gly-TCACAACTA-lys₃
5: TCACAACTA-lys₃

Figure 2. N-Terminally modified PNA. NADI (naphthalene diimide residue) is shown in blue.
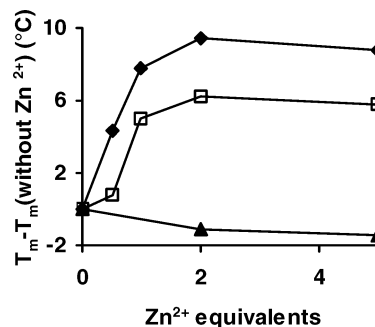

Figure 3. Dependence of melting temperatures of PNA:DNA duplexes on [Zn²⁺]; ◆, 1:6; □, 2:6; ▲, 5:6. [PNA] = [DNA] = 2 μM, [Zn²⁺] = 0, 1, 2, 4, and 10 μM (1−5 equiv), [MOPS] = 10 mM, [NaCl] = 50 mM.

PNA:DNA duplex NADI. A T_m of 1:6 is increased substantially in the presence of Zn²⁺ (Figure 3). The T_m enhancement is higher than the corresponding effect for 2:6 duplex ($\Delta\Delta T_m$ = 2.8−3.5 °C for 2 equiv of Zn²⁺), where L1 is directly attached to the PNA.

Table 1. UV Melting Points of PNA:DNA Duplexes[a]

no.	PNA: DNA	NADI	L[1]	L[2]	$[Zn^{2+}] = 0$	$[Zn^{2+}] = 4\ \mu M$	$\Delta T_m\ °C^b$
		PNA modifiers			T_m, °C		
1	**1:6**	+	+	−	61.1 ± 0.4	70.6 ± 1.7	+9.5
2	**2:6**[9b]	−	+	−	57.2 ± 0.8	63.4 ± 1.1	+6.2
3	**3:6**[10]	+	−	+	72.6 ± 0.7	72.0 ± 1.0	−0.6
4	**4:6**[10]	+	−	−	68.7 ± 1.4	68.0 ± 1.1	−0.7
5	**5:6**	−	−	−	61.0 ± 1.0	60.3 ± 1.5	−0.7
6	**1a:7**	+	+	−	45.0 ± 1.1	57.2 ± 0.4	+12.2
7	**1a:8**	+	+	−	46.7 + 0.7	58.0 ± 1.6	+11.3
8	**1a:9**	+	+	−	45.3 + 1.2	57.2 ± 1.1	+11.9
9	**1a:10**	+	+	−	44.5 + 1.5	56.1 + 0.8	+11.6
10	**1a:11**	+	+	−	c	37.2 ± 0.5	>8.0

[a] Average of at least four melting points ± SD at 2 μM (**1–11, 1a**) strand concentration, MOPS pH 7 10 mM, NaCl 50 mM. [b] Difference between T_m's in the presence and absence of Zn^{2+}. [c] No reversible transition.

Table 2. UV Melting Points of Duplexes of PNA **1a** and **5** with Complementary DNA and DNA Containing Single Mismatches[a]

DNA[b]	$[Zn^{2+}] = 0$	$[Zn^{2+}] = 4\ \mu M$	T_m, °C (PNA **5**) $[Zn^{2+}] = 0$
	T_m, °C (PNA **1**)		
1. ACC AGT GTT GAT TCT	61.1 ± 0.4	70.6 ± 1.7	61.0 ± 1.0
2. ACC **t**GT GTT GAT TCT	59.9 ± 1.1	71.0 ± 1.3	60.2 ± 1.1
3. ACC **c**GT GTT GAT TCT	60.5 ± 1.2	70.8 ± 1.9	59.2 ± 1.4
4. ACC **g**GT GTT GAT TCT	59.9 ± 1.5	70.1 ± 1.4	59.5 ± 1.7
5. ACC A**t**T GTT GAT TCT	53.0 ± 1.9	57.2 ± 1.0	52.9 ± 2.3
6. ACC AG**c** GTT GAT TCT	41.3 ± 1.7	42.5 ± 1.7	40.8 ± 1.7

[a] Conditions are shown in footnote a to Table 1. [b] Mismatched bases are shown as small bold letters.

Thus, cooperative binding of naphthalene diimide and the Zn^{2+} complex is more stabilizing than binding of the Zn^{2+} complex alone. Stabilization of PNA:DNA duplex by the terminal Zn^{2+}-L[1]-NADI residue is more pronounced for a shorter PNA **1a** (+12.2−11.3 °C, entries 6−9, Table 1). This difference in T_m's corresponds to $\Delta\Delta G° = -11.3$ kJ mol^{-1}, $\Delta\Delta H° = -65$ kJ mol^{-1}, $\Delta\Delta S° = -180$ J (mol K)$^{-1}$ for PNA **1a:8** at 298 K.[11] Importantly, the stabilization effect is not dependent on the sequence of the overhang of the DNA target (Table 1). Intercalation of NADI in **1:6** in the way shown in Figure 1B is supported by mismatch discrimination data. In particular, the stability of **1**:DNA duplexes is not dependent on the presence of mismatches in the terminal position (entries 1−4, Table 2). A terminally stacking residue would be expected to reduce fraying of the match terminal base pair and therefore stabilize it over mismatch base pairs.[13] The Zn^{2+}-induced T_m increase for a duplex of **1** with the DNA which has a mutation G→T in the penultimate position is reduced by 5.3 °C in comparison with that for a duplex of **1** with complementary DNA (entries 1 and 5, Table 2). No increase of T_m is observed for duplexes of **1** with DNAs, which have other internal mutations (e.g., entry 6, Table 2). Along with hypochromicity of NADI bands in the absorption spectrum of Zn^{2+}-**1:6**, these data indicate that NADI-L[1]-Zn^{2+} is positioned between the terminal and penultimate base pairs (Figure 1B). In accordance with molecular modeling, intercalation between the second and third base pairs is not possible for steric reasons.

The melting transition of duplex of **1a** with **11** is observed only in the presence of Zn^{2+} (entry 10, Table 1). This example illustrates the possibility to switch binding of PNA probes ON by addition of 2 equiv of Zn^{2+} at room temperature.

The T_m of Zn^{2+}-**1:6** was found to be independent of the time of the sample pre-equilibration before the melting experiment.[11] This indicates that, in mixtures of Zn^{2+}, **1**, and **6**, formation of Zn^{2+}-**1:6** duplex occurs quickly (<5 min[11]). This favorably distinguishes our probes from recently reported Gd^{3+}-dependent DNA probes. In particular, in the presence of 1 equiv of Gd^{3+} (2 μM), 80% conversion of unbound DNAs was achieved after 40 h. A 20-fold excess of Gd^{3+} was required for full conversion.[9a]

In contrast to **1**, binding of L[2]-NADI-gly-PNA (**3**) to **6** is practically independent of Zn^{2+} concentration (entry 3, Table 1). We believe that the equilibrium shown in Figure 1 does not occur in this case, since a pre-organized cyclic ligand L[2] in its free and complexed form has a similar structure and a similar charge. As is evident from the high T_m of **3:6** and its Zn^{2+} complex, both have **B** conformation in solution (Figure 1B).

At pH 7, the neutral form of L[1] is expected to predominate[14] and is converted to a dicationic substituent on a complexation with Zn^{2+}. Therefore, a T_m increase of 6.2−12.2 °C for the **1:6**, **2:6**, **1a**:7−11 duplexes may be explained by electrostatic interactions between $(Zn-L^1)^{2+}$ and negatively charged DNA backbone. L[2] is already present in diprotonated form at pH 7; therefore, in agreement with the electrostatic model, DNA binding by L[2]-NADI-gly-PNA conjugate is independent of Zn^{2+}.

In summary, micromolar Zn^{2+} increases the affinity of the PNA probes to single-stranded DNA significantly, which is reflected in the best case in a 12.2 °C increase of T_m upon addition of Zn^{2+}. These probes hybridize to DNA within a few minutes at 22 °C.

Supporting Information Available: Details of synthesis, UV-melting, and fluorescence experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) L[1] belongs to compounds of the type acceptor-CH$_2$N(picolyl)$_2$. Therefore, it may be estimated that the pK_1 of L[1] is close to that of tris(picolyl)-amine (pK_1 = 6.2: ScQuerry Vn 4.06, IUPAC stability constants database).

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