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Zn^{2+} dependent DNA binders based on terminally modified peptide nucleic acids

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Abstract—Two ligand–intercalator–peptide nucleic acid conjugates (L-NADI-PNAs) have been synthesized. Affinity of these conjugates to their complementary DNAs was found to be affected by Zn^{2+}. The magnitude of this effect could be controlled by a variation of the ligand. Upon binding Zn^{2+} the L-NADI-PNAs form positively charged ZnL complexes, which interact with the negatively charged DNA backbone. This electrostatic interaction stabilizes PNA/DNA duplexes. It has been found that Zn^{2+} dependent stabilization takes place only if the ZnL complex has a higher total positive charge than the ligand. Linear correlation has been observed between Zn^{2+} induced stabilization of PNA/DNA duplexes and difference of charges of the ZnL complex and the ligand.

Peptide nucleic acid (PNA) is a synthetic DNA analogue, in which a negatively charged phosphodiester backbone is substituted for a neutral polyamide backbone. Due to this modification, PNA binds nucleic acids with higher affinity and, in most cases, specificity.[1] In contrast to DNA, PNA is stable in the presence of enzymes and can potentially be used for gene regulation in vivo.[2] Binding of PNA to nucleic acids is only weakly dependent upon Zn^{2+} concentration, while terminally modified PNAs carrying some special ligands show significant sensitivity toward this metal ion.[3] This property can be used for the design of antisense agents specific for cells containing elevated amounts of Zn^{2+}, for example, nerve,[4] sperm,[5] and some cancer cells.[6] It has been found that malaria parasite (*Plasmodium falciparum*) infecting red blood cells accumulates Zn^{2+}. Concentration of free (chelatable) Zn^{2+} in these parasites is increased up to 50-fold in comparison with the host erythrocyte.[7] Therefore, Zn^{2+}-sensitive PNAs could be potentially used as drugs against malaria infections.

Recently, we have demonstrated that binding affinity of L-NADI-PNAs (L: bis-(2-pyridylmethyl)amine, cyclam or 1,4,9-triazacyclononane, NADI: 1,4,5,8-naphthalenetetracarboxylic acid diimide) is substantially increased in the presence of micromolar Zn^{2+}.[3b] Zn^{2+} ions control intercalation of NADI within PNA/DNA duplex via binding to L (Fig. 1). Herein we present the results of a further variation of the ligand structure in the L-NADI-PNAs. The ligands reported in our first communication form +2 charged ZnL complexes and are either neutral (bis-(2-pyridylmethyl)amine) or +2 positively charged (cyclam, 1,4,9-triazacyclononane: LH_2^{2+}) in their free state at pH 7. Now we have chosen chelating ligands, which are neutral at pH 7, expected to bind micromolar [Zn^{2+}] and lose one proton upon Zn^{2+} coordination forming monocharged ZnL complexes: L1 and L2 (Scheme 1). This completes a series of the L-NADI-PNA conjugates, in which the formal PNA charge is alternating between 0 and 2 units upon Zn^{2+} complexa-

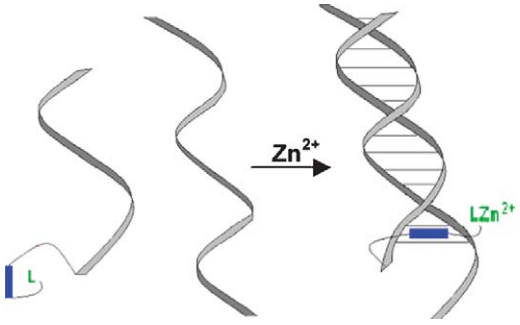

Figure 1. Zn^{2+} dependent binding of PNA probes to DNA. Naphthalene diimide, NADI is shown as a blue thick stick, L is a metal binding ligand.

Keywords: Peptide nucleic acids; Zinc; DNA; Binding.
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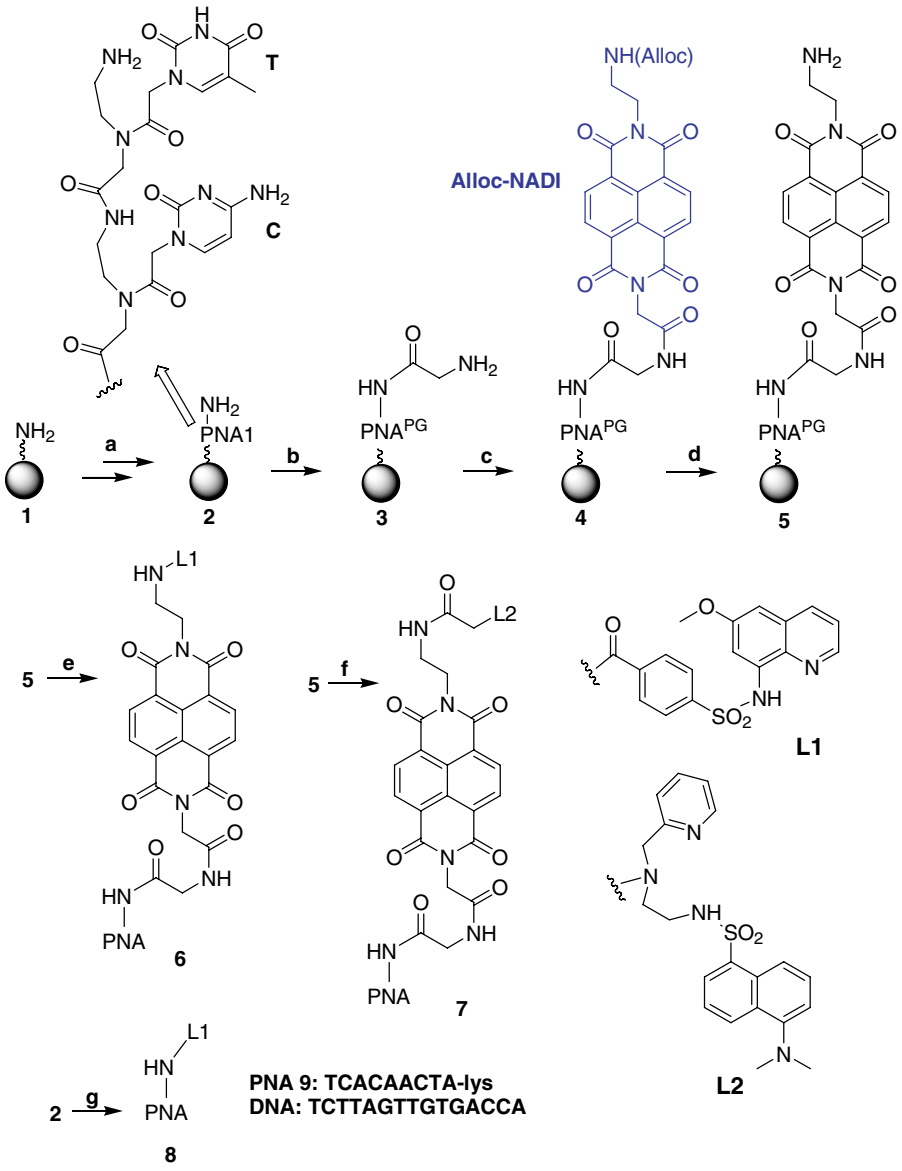
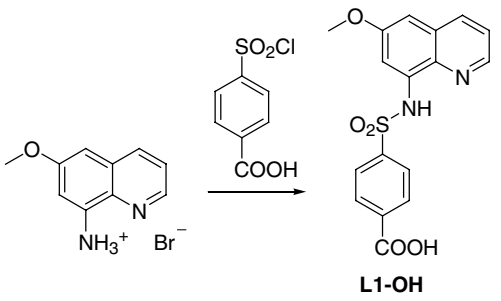


Scheme 1. Synthesis of PNAs **6**, **7** and **8**. Reagents: (a) PNA synthesis,[8] (b) Fmoc-Gly-OH, HBTU, HOBT, DIEA, DMF, (c) Alloc-NADI-OH, HBTU, HOBT, DIEA, DMF, (d) [Pd(PPh3)4], PPh3, $(NEt_2H_2)(HCO_3)$, CH_2Cl_2, (e) 1—*N*-(6-methoxy-8-quinoyl)-4′-carboxyl-benzensulfonamide (L1-OH), HBTU, HOBT, DIEA, DMSO; 2—TFA, *m*-cresol, (f) 1—bromoacetyl bromide, DIEA, DMF; 2—*N*-(2-pyridylmethyl)-*N*′-dansyl-1,2-ethanediamine, DIEA, DMF, 3—TFA, *m*-Cresol, (g) 1—*N*-(6-methoxy-8-quinoyl)-4′-carboxyl-benzensulfonamide (L1-OH), HBTU, HOBT, DIEA, DMSO; 2—TFA, *m*-cresol.

tion. Testing-binding affinity of PNAs in this series can clarify the mechanism of Zn^{2+} induced stabilization of L-NADI-PNA/DNA duplexes. This can help in further optimization of PNA probes, which can be switched on by Zn^{2+}. If electrostatic interactions between the ZnL complex and the phosphodiester backbone of the DNA play a dominant role, stability of L-NADI-PNA/DNA duplexes with the new ligands is expected to be between that of the corresponding duplexes with L = bis-(2-pyridylmethyl)amine and cyclam. The structure of L should, in this case, be less important.

L1 is an analogue of the known fluorescent Zn^{2+} indicator, TSQ.[4a,9] For its conjugation to the PNA N-terminus we have introduced a carboxylic group at *para*-position of its phenyl ring, L1-OH. L1-OH ligand was synthesized by the reaction of *p*-carboxyphenylsulfonyl chloride with commercially available 8-amino-6-methoxyquinoline hydrobromide in pyridine (Scheme 2). Identity of this compound has been confirmed by NMR spectroscopy and X-ray analysis.[10,11] L1-OH is crystallized as a pyridinium salt. As expected, 8-aminoquinoline fragment, which is responsible for Zn^{2+} binding, is fully planar. In a conformation found in the crystal structure, the coordination place for Zn^{2+} is stericly hindered by the *p*-carboxyphenyl residue. However, in solution the latter fragment can flip away from the coordination site by rotation along single S1–N1 bond.[12] In ESI-mass spectrum of 2:1 and 1:1 mixtures of L1-OH (100 μM) and Zn^{2+}, a strong peak corre-



Scheme 2. Synthesis of L1-OH.

sponding to $[ZnL_2]^-$ and a weaker peak corresponding to $[Zn(L)(SO_4)]^-$ are observed (Fig. 2). In the presence of Zn^{2+} excess (L1-OH/Zn^{2+} = 1/5) intensity of peaks corresponding to ZnL complexes ($[Zn(L)Cl]^-$ and $[Zn(L)Cl_2]^-$ ions) is increased relative to that of peaks corresponding to $[ZnL_2]^-$. Fluorescence intensity of L1-OH (100 μM) is substantially increased upon addition of up to 0.5 equiv Zn^{2+} (Fig. 3). Further addition of the metal ion leads to gradual fluorescence quenching (down to 42% relative to fluorescence intensity in the presence of 0.5 equiv Zn^{2+}). Both mass spectral and fluorescence data are in agreement with the initial formation of ZnL_2 complex and its further transformation to ZnL at higher concentrations of the metal ion. We could not study the interaction between Zn^{2+} and L1-OH at lower concentrations using fluorescence and UV–vis spectroscopy, because L1-OH has rather low fluorescence quantum yield and extinction coefficient. Analogue of L1-OH, 8-(methanesulfon-amido)quinoline at 100 μM, pH 7, in the presence of equimolar Zn^{2+} forms both ZnL (71%) and ZnL_2 (29%) complexes, while at 1 μM it forms only ZnL complex (98%).[13] It is reasonable to suggest that formation of ZnL complex

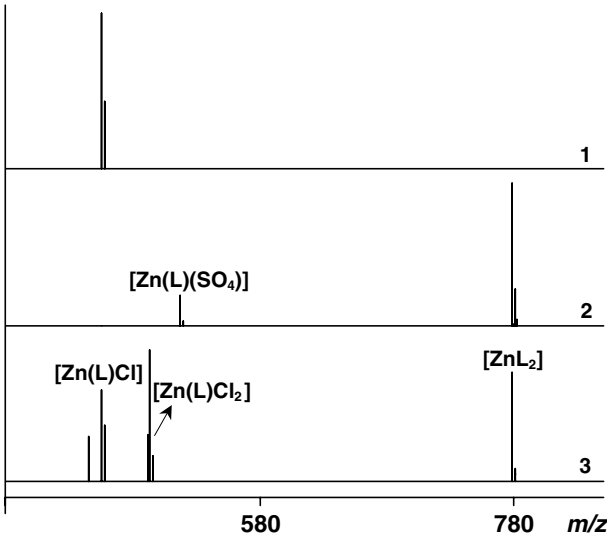


Figure 2. ESI mass spectra of mixtures containing L1-OH and $ZnSO_4$ in DMSO (1%)/aqueous triethylammonium acetate (pH 7, 1 mM, 10%)/AcCN. 1: L1-OH 10 μM and $ZnSO_4$ 10 μM; 2: L1-OH 100 μM and $ZnSO_4$ 100 μM; 3: L1-OH 100 μM and $ZnSO_4$ 500 μM.

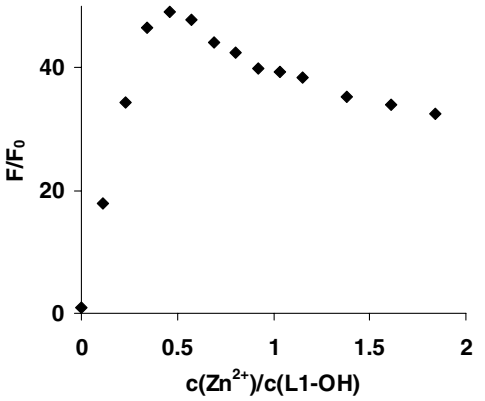


Figure 3. Fluorescent titration of L1-OH (0.1 mM) by $ZnSO_4$ in aqueous MOPS (10 mM), NaCl (50 mM) buffer, pH 7.

will be also more favored at lower concentrations of L1-OH. This could be confirmed by mass spectral study. In particular, ratio of intensities of peaks corresponding to $[Zn(L)Cl]^-$ and $[ZnL_2]^-$ is dramatically increased upon dilution of a mixture of Zn^{2+} and L1-OH from 100 to 10 μM (Fig. 2).

The other ligand, L2-H, has been studied earlier as a polymer bound fluorescent sensor for Cu^{2+}.[14] We have found that fluorescence of L2 is also affected by Zn^{2+} in a concentration dependent manner. On the basis of the L2-H titration by Zn^{2+}, we could determine that at micromolar [L2-H] only ZnL complex is formed, $\log K = 6.2 \pm 0.2$ (Fig. 4). Formation of this complex could be confirmed by mass spectrometry. The NH group of sulfonamide ligands is usually deprotonated upon Zn^{2+} coordination. Therefore, one can expect that positively charged $[ZnL]^+$ complexes will be formed in highly dilute solutions of Zn^{2+} and PNA conjugates modified with either L1 or L2 ligands.

L-NADI-PNA conjugates with L = L1 or L2 have been synthesized in accordance with Scheme 1 (PNAs **6** and **7**). First, the PNA1 portion was prepared using solid phase synthesis. Further glycine residue has been

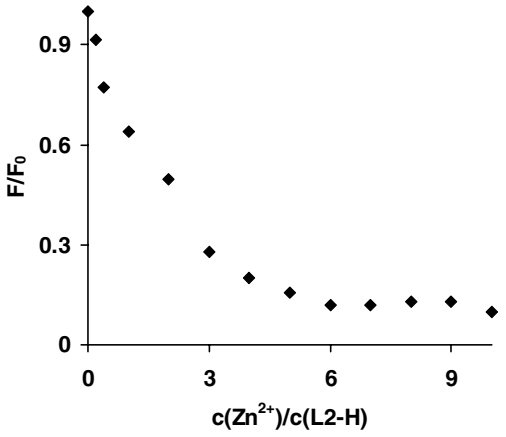


Figure 4. Fluorescent titration of L2-H (1 μM) by $ZnSO_4$ in aqueous MOPS (10 mM, pH 7), NaCl (50 mM).

attached by coupling Fmoc-Gly-OH pre-activated by HBTU/HOBT mixture and deprotecting α-amino group using piperidine. Alloc-NADI-OH has been coupled under similar conditions. Its amino group has been deprotected using $[Pd(PPh_3)_4]$, PPh_3, $(NEt_2H_2)(HCO_3)$ mixture (PNA **5**). Finally, L1-OH has been attached to the terminal amino group, resulting in PNA **6** deprotected by TFA/*m*-cresol mixture and purified by HPLC. For the synthesis of PNA **7** the amino group of PNA **5** has been acylated by bromoacetyl bromide and the resulting bromoalkyl ~ PNA has been aminated using *N*-(2-pyridylmethyl)-*N*′-dansyl-1,2-ethanediamine (L2-H). PNA **8** has been synthesized by direct conjugation of L1-OH with unmodified PNA **2** using HBTU/HOBT activating mixture. PNAs **5** and **8** have been cleaved from solid support, deprotected and purified analogously to PNA **6**. Purity of the obtained PNAs has been higher than 90% in accordance with HPLC and MALDI-TOF MS analysis.

A conjugate of L1 and PNA (PNA **8**, Scheme 1) binds its complementary DNA slightly weaker than the corresponding unmodified PNA (entries 7, 11, Table 1). Upon addition of up to 1 equiv Zn^{2+} T_m of the PNA **8**/DNA duplex (2 μM) is increased by 3.4 °C, while further Zn^{2+} additions lead to its slight destabilization. This indicates the formation of stable 1:1 Zn^{2+}/PNA **8** complex. Stability of unmodified PNA/DNA duplexes is slightly decreased in the presence of Zn^{2+} (entries 11–13, Table 1). Both L1-NADI and L2-NADI modifications stabilize PNA/DNA duplexes, as it follows from their elevated melting points (ΔT_m = +5.8 and +5.1 °C correspondingly, relative to the unmodified duplex T_m, entries 1, 3, and 7, Table 1). However, NADI alone has a stronger effect on the duplex stability, ΔT_m = +7.7 °C.[15] In comparison with the NADI modification the L-NADI ones are correspondingly ΔT_m = −1.9 and −2.8 °C less stabilizing. Destabilizing ligand effect has been also observed for bis-(2-pyridyl)amine (ΔT_m = −7.6 °C), while both cyclam and 1,4,9-triazacyclononane provide additional duplex stabilization (ΔT_m = +3.9 °C).[3b,15] At pH 7 all three ligands showing the destabilizing effect are neutral, while the stabilizing ligands are positively charged. This allows suggesting that electrostatic interactions of L with negatively charged DNA backbone define at least in part the duplex stability.

Table 1. UV melting points of PNA:DNA duplexes[a]

Entry	PNA	PNA modifiers		c(Zn^{2+}) (μM)	T_m (°C) of PNA/DNA
		NADI	L		
1	**6**	+	L^1	0	53.3 ± 0.7
2	**6**	+	L^1	1–10	[b]
3	**7**	+	L^2	0	52.6 ± 0.4
4	**7**	+	L^2	1	54.5 ± 0.9
5	**7**	+	L^2	2	56.5 ± 1.2
6	**7**	+	L^2	10	55.9 ± 0.9
7	**8**	–	L^1	0	45.8 ± 0.8
8	**8**	–	L^1	1	46.6 ± 0.9
9	**8**	–	L^1	2	49.2 ± 0.2
10	**8**	–	L^1	10	47.4 ± 0.9
11	**9**	–	–	0	47.5 ± 1.4
12	**9**	–	–	2	46.7 ± 1.1
13	**9**	–	–	4	45.9 ± 0.6

[a] Average of at least four melting points; strand concentration 2 μM, MOPS 10 mM, pH 7, NaCl 50 mM.
[b] Irreversible melting transitions.

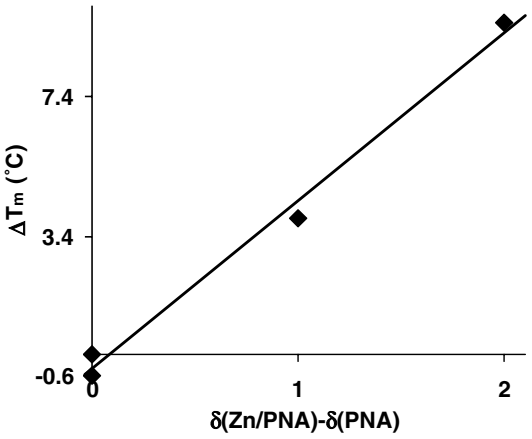


Figure 5. Correlation between Zn^{2+} induced PNA/DNA duplex stabilization (ΔT_m) and changes of formal charge of the PNA upon metal ion coordination (δ(Zn/PNA)–δ(PNA)). PNA = L-NADI-PNA, L = cyclam, 1,4,9-triazacyclononane, L2, bis-(2-pyridylmethyl)amine).

In the presence of Zn^{2+}, formation of L1-NADI-PNA/DNA duplex is irreversible even at very slow cooling rates (0.1 °C/min). Therefore, accurate determination of melting points was not possible in this case. Stability of L2-NADI-PNA/DNA is increased upon addition of up to 1 equiv Zn^{2+} (ΔT_m = +3.9 °C). Further additions lead to slight destabilization of the duplex. This indicates that 1:1 Zn^{2+}/L2-NADI-PNA complex is formed at our experimental conditions, which is in agreement with the studies of L2-OH interaction with Zn^{2+} using fluorescent titration and mass spectrometry. It should be noted that overall Zn^{2+} effect on melting points of L-NADI-PNA/DNA duplexes is weaker with L = L2 (ΔT_m = +3.9 °C) than that with previously studied L = bis-(2-pyridyl)amine (ΔT_m = +9.5 °C).[3b] This may be explained by different charges of 1:1 complexes with the corresponding ligands. In particular, Zn(L1) has +1 formal charge, while Zn(bis-(2-pyridylmethyl)amine) has +2 formal charge. Strength of the interaction of the latter complex with negatively charged DNA backbone is expected to be stronger. Within 4 studied examples of L-NADI-PNAs a linear correlation has been found between Zn^{2+} induced PNA/DNA duplex stabilization and changes of the formal charge of the PNA upon metal ion coordination (Fig. 5). This indicates that electrostatic interactions between the modifier of the PNA and charged backbone of the DNA play a dominating role in determining stability of the studied PNA/DNA duplexes.

Acknowledgments

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Supplementary data

Description of synthesis of L1-OH and modified PNAs. Molecular structure of L1-OH. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.02.002.

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