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Synthesis and DNA Binding Properties of Terminally Modified Peptide Nucleic Acids

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Abstract—PNAs with terminal modifications of varying structure and charge were synthesized and their binding to DNA was studied. A variation in thermal stability of **19**. 8 °C has been observed between the least and the most stable PNA–DNA duplexes. The most stable duplex melts 7.7 °C higher than the duplex of the corresponding non-modified PNA and complementary DNA. It has been shown that sequence fidelity of the PNA conjugate having the highest DNA affinity is significantly better than that of non-modified PNA. The results obtained can be used for the design of PNA probes, whose binding to DNA is sequence independent.

Peptide nucleic acids (PNA) are DNA analogues in which the sugar-phosphate backbone has been substituted by *N*-(2-aminoethyl)-glycine units. PNA is characterized by its high chemical stability and nucleic acid binding specificity. Therefore, PNAs are widely used as probes in hybridization based applications, for example, in PNA-arrays (chips), in situ hybridization, as PNA biosensors, in nucleic acids purification and as antisense agents.[1] However, the efficiency of binding of PNA to DNA is dependent on the purine content of the PNA strand as well as on the GC content of the PNA–DNA duplex. Therefore, stability of duplexes of pyrimidine rich PNA probes and fully complementary DNA is in many cases comparable to stability of duplexes of purine rich PNA probes with non-complementary DNA.[2] In this case, the hybridization event does not correlate with the Watson–Crick pairing rules and a hybridization assay gives false positive and negative results. Herein we report the results of an exploratory study aimed at finding terminal PNA modifications, which affect the binding affinity of PNA towards DNA. Using these modifications one can equalize stabilities of duplexes of DNA with different PNA to obtain PNA probes, whose hybridization with DNA is sequence independent. An alternative strategy including the incorporation of non-natural bases into the interior of PNA template has proven to be successful for controlling the stability of PNA–DNA duplexes, but it often includes laborious synthesis.[3] N-terminal modifications affecting PNA–DNA duplex thermal stability substantially have been reported by Okamoto et al. for 8-methoxypsoralen and Harrison et al. for polycationic peptides (for both $\Delta T_m = +8.0\,^{\circ}C$).[4]

We have tested the effects of terminal PNA-modifications of different classes on the stability of PNA–DNA duplexes (Scheme 1): (a) intercalators, which are neutral (α, β, ε), negatively (γ) or positively charged (ϕ), dimeric intercalators and intercalators attached to both C- and N-termini of PNA, (b) the positively charged, non-aromatic fragment (χ) capable of stabilizing PNA–DNA duplexes due to electrostatic interactions with negatively charged backbone of the DNA, and (c) a cholic acid residue (δ), which can stabilize the duplexes by means of hydrophobic interactions with a terminal basepair.[5,6]

Conjugation of amines to PNA has been accomplished by subsequent coupling of a bromoacetyl group using bromoacetyl bromide and its amination using an excess of amine (Scheme 2).[7] Modifications available as carboxylic acid building blocks were coupled with PNA using HBTU, HOBT, DIEA coupling mixture analogously to the reported procedure (Scheme 2).[8] Acid ϕ-OH, **8** was synthesized and characterized in these laboratories.[9] In the case of cholic acid, δ-OH, the conversion of the starting material was found to be complete within 20 min, though free hydroxyl groups of the desired product were overacylated with cholic acid residues.

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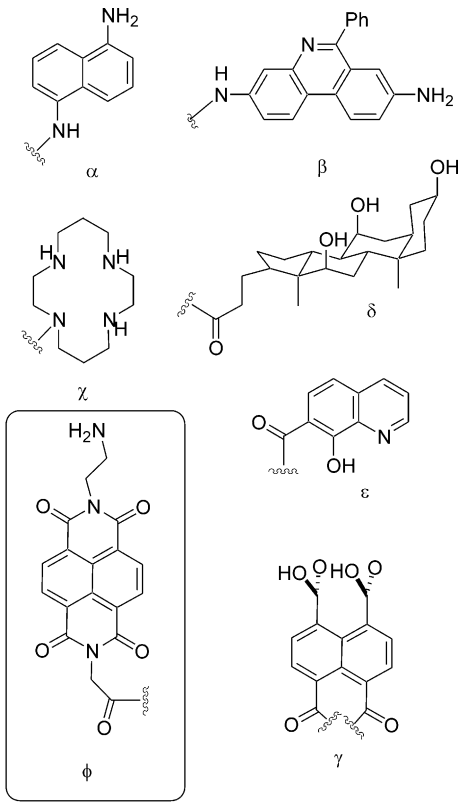
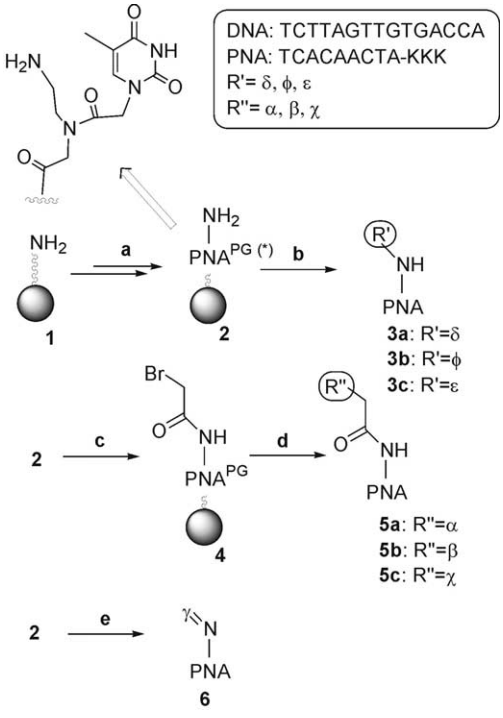
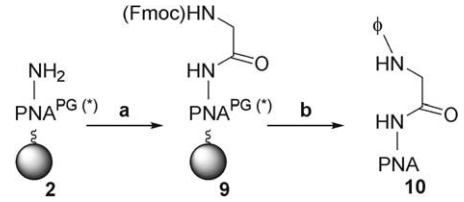


Scheme 1. Modifiers of termini of PNA.

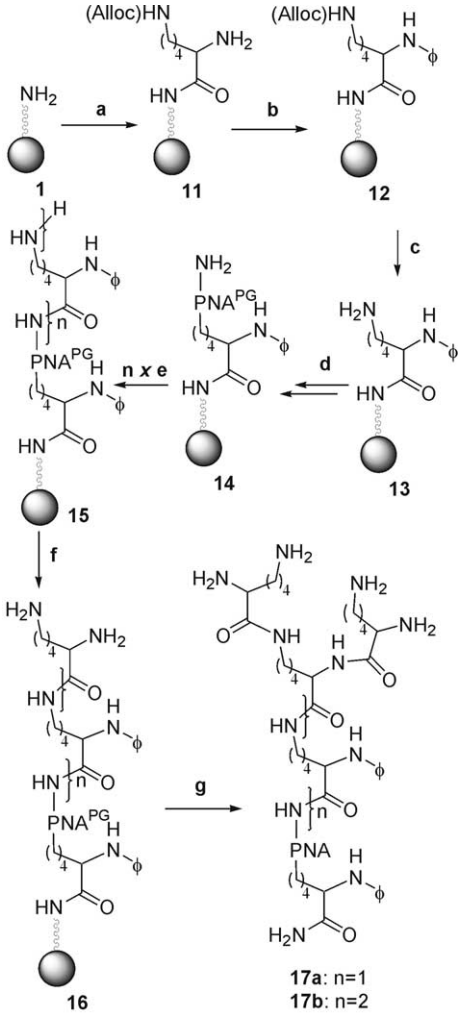


Scheme 2. (a) PNA synthesis; (b) (1) R′-OH, HBTU, HOBT, DIEA, DMF; (2) TFA, *m*-cresol for R′ = ϕ, ε and (2) NH_3 (aq) 25%; (3) TFA, *m*-cresol for R′ = δ; (c) Bromoacetyl bromide, DIEA, DMF; (d) (1) R″-H, DIEA, CH_2Cl_2 for R″ = χ and R″-H, DIEA, DMF for R″ = α, β; (2) TFA, *m*-cresol; (e) (1) 1,4,5,8-Naphthalene-tetracarboxylic dianhydride, DIEA, pyridine; (2) TFA, m-cresol, H_2O. (*) PG = Bhoc for PNA nucleobases and BOC for ε-amino group of Lys.

Treatment with 25% aqueous ammonia for 24 h gave the single product, **3a**. PNA **6** (Scheme 2) was synthesized by reaction of 1,4,5,8-naphthalenetetracarboxylic dianhydride with the terminal amino group of PNA followed by deprotection in TFA/*m*-cresol/water (4/1/0.5) solution for 24 h at 22 °C. Conjugate **10** was synthesized by attaching Fmoc-Gly-OH to PNA first, then deprotecting the amino group and finally coupling ϕ-OH (Scheme 3). Two and three naphthalene diimides were conjugated to PNA (Scheme 4) by attaching Fmoc-Lys(ε-Alloc)-OH to the Rink resin, followed by



Scheme 3. (a) Fmoc-Gly-OH, HBTU, HOBT, DIEA, DMF; (b) (1) piperidine, DMF, (2) ϕ-OH, HBTU, HOBT, DIEA, DMF, (3) TFA, *m*-cresol.



Scheme 4. (a) (1) (Fmoc)Lys(ε-Alloc)-OH, HBTU, HOBT, DIEA, DMF, (2) piperidine, DMF; (b) **10**, HBTU, HOBT, DIEA, DMF; (c) $Pd(PPh_3)_4$, PPh_3, $(NEt_2H_2)(HCO_3)$, CH_2CH_2; (d) PNA synthesis; (e) (a) + (b) + (c); (f) (1) (Fmoc)Lys(Fmoc)-OH, HBTU, HOBT, DIEA, DMF, (2) piperidine, DMF; (g) (1) (f), (2) TFA, *m*-cresol.

Fmoc deprotection and coupling of ϕ-OH, **8**. The reaction was continued by Alloc cleavage using a $Pd(PPh_3)_4$ catalyst[11] and PNA synthesis off the deprotected ε-amino group of Lys. After one more cycle of coupling of Fmoc-Lys(ε-Alloc)-OH to the N-terminus of the PNA, Fmoc deprotection, coupling of ϕ-OH, **8**, and Alloc cleavage, the material obtained was split into two equal parts. One of these was used for synthesis of **17a** by conjugation of a small polyamine dendrimer, which was to provide for solubility of the final product. The other portion was used for synthesis of **17b** by repeating the cycle for coupling of **8**: Fmoc-Lys(ε-Alloc)-OH attachment, Fmoc deprotection, coupling of **8** and dendrimer coupling. All synthesized PNAs were HPLC purified and their identity was confirmed by MALDI-TOF mass spectrometry (Table 1).

Melting points of PNA–DNA duplexes are listed in Table 1. The intercalators, 1,5-diaminonaphthalene, α, and 3,8-diamino-6-phenyl-phenanthridine, β, have destabilizing or weakly stabilizing effects correspondingly (entries 1,2, Table 1), while ϕ and ε stabilize PNA–DNA duplexes more significantly in comparison with non-modified PNA–DNA (entries 6, 7, 12, Table 1). Cholic acid residue, δ, affects affinity of PNA towards DNA weakly (ΔTm = 3.5 °C), which is in contrast to DNA-cholic acid conjugates substantially increasing thermal stability of DNA duplexes (+8–11 °C).[5] This is in line with the observation that ligands having high affinity towards DNA duplex do not necessarily bind well to PNA–DNA duplex.[12] The dianionic fragment γ destabilizes PNA–DNA ($\Delta T_m = -12.1$ °C), which is in agreement with data for conjugates of PNA and analogously charged fluorescein ($\Delta T_m = -12.0$ °C).[13] Since positively charged residues normally stabilize PNA–DNA due to the electrostatic interaction with the negatively charged DNA backbone,[1] it was surprising that the dicationic at pH 7 macrocycle, χ, does not alter PNA binding affinity towards DNA at the low salt conditions used in T_m-measurements.

Of the most stabilizing residues ϕ, ε, and δ we have chosen ϕ for further binding optimization. The conjugates of ε and δ were difficult to handle due to their strong absorption on the surfaces of reaction vials and optical cells.

The naphthalene diimide residue, ϕ is an efficient intercalator of DNA duplexes.[14] Although an effect of conjugation of ϕ with PNA was not studied before, it has been recently shown that the smaller intercalator, naphthalene imide is as stabilizing as a terminal $G^{PNA}C^{DNA}$ basepair (4–5 °C) when attached to N-terminus of PNA via a–CH_2–C(O)–Gly-linker.[15] With the shorter linker (–CH_2–C(O)–) naphthalene diimide, ϕ, stabilizes PNA–DNA duplex weaker (+2.5 °C). This indicates that –CH_2–C(O)– might be a too short linker for optimal interaction of ϕ with PNA–DNA duplex. Indeed, conjugate **10** having a longer linker binds DNA much stronger as it can be seen from the higher melting point of **10**:DNA ($\Delta T_m = +7.7$ °C). Dimeric naphthalene diimides are reported to be even stronger binders of DNA duplexes than corresponding monomers.[16] Therefore, PNA containing several ϕ units were synthesized. Surprisingly, only a minor gain in DNA affinity was achieved upon conjugation of two and three residues ϕ to PNA in comparison with conjugate **3b** (entries **10**, **11**, Table 1). Both conjugates bind DNA weaker than conjugate **10**. This can be explained by the stabilization of the single stranded PNA or its aggregation in solution due to the hydrophobic interactions between the ϕ units. In agreement with this is the observation that at the conditions of melting experiments (Table 1, footnote) the extinction coefficients of the absorption bands of ϕ in the conjugates **3b**, **17a**, and **17b** are not linearly dependent upon the number of ϕ-units in the conjugates: $2*\varepsilon^{382}$(**3b**)/ε^{382}(**17a**) = 1.04, $3*\varepsilon^{382}$(**3b**)/ε^{382}(**17b**) = 1.3. Similar effects were reported for the dimers of naphthalene diimide.[16]

Sequence fidelity of PNA is usually high[1]. For example, the duplex of the non-modified PNA used in this study and DNA with a single mismatch in its interior (**DNA2**, T→C) melts 24.8 °C lower than the corresponding duplex with fully matched DNA, **DNA1**:**PNA** (Table 1, entries 12,13). Interestingly, duplex of **DNA2** and

Table 1. Properties of PNA conjugates

No.	Duplex[a]/PNA-modification	T_m (°C)[b]	ΔT_m	PNA mass $[M+H]^+$ m/z	
				Found	Calcd
1	**DNA1**:**5**/α	57.9±1.3	−3.1	2988.1	2988.0
2	**DNA1**:**5b**/β	61.6±0.6	+0.6	3114.2	3115.2
3	**DNA1**:**5c**/χ	60.8±1.8	−0.2	3028.9	3028.5
4	**DNA1**:**6**/γ	48.9±0.2	−12.1	3058.2	3057.8
5	**DNA1**:**3a**/δ	64.5±0.5	+3.5	3179.2	3180.4
6	**DNA1**:**3c**/ε	63.5±1.1	+2.5	2958.5	2959.9
7	**DNA1**:**3b**/ϕ	63.5±1.1	+2.5	3138.5	3137.8
8	**DNA1**:**10**/ϕ	68.7±1.4	+7.7	3194.8	3194.4
9	**DNA2**:**10**/ϕ	34.1±1.9	−26.9	—	—
10	**DNA1**:**17a**/2×ϕ	64.9±0.5	+3.9	3744.6	3744.0
11	**DNA1**:**17b**/3×ϕ	64.4±1.5	+3.4	4223.9	4222.2
12	**DNA1**:PNA	61.0±1.0	0	2786.0	2788.3
13	**DNA2**:PNA	36.2±1.5	−24.8	—	—

[a]**DNA1** TCTTAGTTGTGACCA, **DNA2** TCTTAGTCGTGACCA.
[b]Melting and cooling curves were recorded on a Varian Cary 100 Bio UV–vis spectrophotometer measuring absorbance at 260 nm in a 1 cm path length at heating/cooling rate 0.5 °C/min in MOPS buffer (10 mM, pH 7), NaCl (50 mM), [PNA] = [DNA] = 2 μM.

conjugate **10**, which is the strongest DNA binder found in this work, is slightly less thermally stable and melts 26.9 °C lower than **DNA1**:**PNA** (Table 1, entries 9,12). Since **DNA1**:**10** melts at considerably higher temperature than **DNA1**–**PNA**, conjugate **10** discriminates between match (**DNA1**) and mismatch (**DNA2**) DNA sequences better ($\Delta T_m = 34.6$ °C, Table 1, entries 8,9) than non-modified PNA does ($\Delta T_m = 24.8$ °C, Table 1, entries 12,13).

Stabilizing effect of naphthalene diimide, ϕ, was reproduced for analogues of conjugate **10**, having different PNA sequences: AACTA, GGAACTA, CTACAACTA, and ATACAACTA. Experimental details will be reported in a full paper.

In summary, we have synthesized PNA conjugates containing fragments of different structure and charge, and studied their binding to DNA. The variation of thermal stability of PNA–DNA duplexes between the least and the most stabile duplexes is 19.8 °C. The strongest DNA binder found in this study shows high sequence fidelity. The results obtained can be used for the design of PNA probes whose binding to DNA is sequence independent.

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7. Final cleavage using TFA/m-cresol 4/1 (100 μL for 2 μmol PNA, 90 min, at room temperature); PNA workup: PNA solution in TFA/m-cresol is filtered, mixed with 20 volumes of diethylether and the precipitate formed is filtered, washed with diethylether and air dried. The white solid is dissolved in water (200 μL) and HPCL purified.
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9. Synthesis of ϕ-OH, **8**. 1,4,5,8-Naphthalenetetra-carboxylic dianhydride (3.35 g, 12.5 mmol) was suspended in pyridine (65 mL) and added to Wang resin loaded with H-Gly-residues[10] (5g, 4.5 mmol of NH_2-groups). The mixture was heated to 65 °C and DIEA (4.4 mL, 25 mmol) was added. The slurry obtained was refluxed for 8 h, then filtered hot, washed with hot (90 °C) pyridine, DMF, CH_3CN and dried. Then TFA in CH_2Cl_2 (20%, 60 mL) was added. After 3 h, the slurry was filtered and the resin washed with CH_2Cl_2 (30 mL). The filtrates were combined, volatiles evaporated, and diethylether (200 mL) was added to the solid residue. The solid was filtered, washed with diethylether and dried to give N-(carboxymethyl)-1,4,5,8-naphthalenetetracarboxylic anhydride-imide, **7**, 0.50 g, 46%. LDI-TOF MS (negative mode) *m*/*z* found 325.9, calcd for $C_{16}H_6NO_7$ $[M\cdot]^-$ 325.2. ^1H NMR (200 MHz, DMSO-d_6): δ 8.60 (s, 4H), 4.67 (s, 4H). N-*t*-Butyloxycarbonylethylenediamine (0.70 g, 1.5 mmol), DIEA (0.7 mL, 4.0 mmol) and **7** (0.50 g, 1.5 mmol) were mixed together in hot pyridine (80 mL, 65 °C) and the resulting suspension was stirred at this temperature for 8 h. Then volatiles were evaporated (10 mbar, 40 °C), aqueous HCl solution (1%, 50 mL) added, and the precipitate formed was filtered, washed with ice water (10 mL), and dried to give **8**, 0.60 g (yield 81%). LDI-TOF MS (positive mode) *m*/*z* found 467.4, calcd for $C_{23}H_{21}N_3O_8$ $[M\cdot]^+$ 467.6. TLC in ethanol/AcOH/CH_2Cl_2 (5/2/97): R_f 0.6. ^1H NMR (200 MHz, DMSO-d_6, at 60 °C): δ 8.57 (m, 4H), 6.81 (t, 1H), 4.60 (s, 2H), 4.05 (t, 2H), 3.18 (m, 2H), 1.10 (s, 9H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 169.8, 163.4, 163.0, 156.6, 132.0, 131.7, 131.1, 127.7, 127.1, 126.8, 126.1, 78.3, 53.4, 28.8. UV–vis: λ_{max} (nm) 381, 361, 344 shoulder.
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