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Hybridization dependent cleavage of internally modified disulfide–peptide nucleic acids

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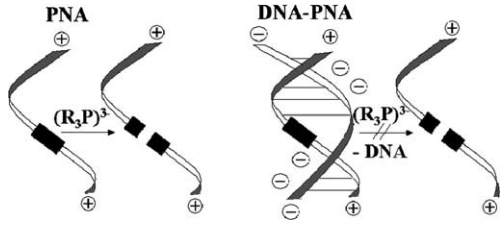
Abstract—Selectivity of the cleavage of single stranded over hybridized forms of internally modified disulfide–peptide nucleic acids (PNA) has been optimized using a series of phosphines and thiols, which have different sizes and charges. For the most selective cleaver found (tris-(carboxyethyl)-phosphine), reactivity of single stranded PNA is 33 times higher than that of the PNA–DNA duplex. Selectivity of single stranded disulfide–PNA cleavage has been explained in terms of electrostatic interaction between the substrate and the cleaver.

Nonenzymatic nucleic acid triggered ligation reactions are attracting much attention due to their potential use in nucleic acid analysis.[1] Reactions of nucleic acids, which are inhibited rather then triggered by their complementary strands, can also be used in analysis. In particular, it has been demonstrated that selective cleavage of unbound DNA probes catalyzed by single strand specific nucleases[2] can be applied to mass spectrometric detection of PCR amplified DNA.[3] This enzymatic approach cannot be applied to nucleic acid analogues, for example, peptide nucleic acids (PNA)[4] and heavily modified nucleic acid probes since they are not substrates of nucleases. The other disadvantage is that DNA desalting is required for analysis.

In this letter we describe a nonenzymatic reaction, in which cleavage of disulfide group in internally modified PNA by an external reductant is strongly inhibited by complementary DNA (Scheme 1). This chemical transformation can be viewed as the analogue of DNA cleavage by single strand specific nucleases in a way that in both the enzymatic and the chemical reactions single stranded substrates are more active. This reaction can potentially substitute nuclease catalyzed DNA hydrolysis in nucleic acids analysis.[3] Due to high affinity and selectivity of PNA binding to single stranded DNA (ssDNA), PNA based assays have some advantages,



Scheme 1. Cleavage of disulfide group in PNA interior in the presence and absence of complementary DNA.

which include, for example, higher sequence fidelity and possibility to use PCR amplified DNA duplexes directly rather than separated ssDNA.[5] Moreover, the assays based on PNA–DNA binding can be conducted in salt free conditions, which makes detection easier.[4]

In single stranded form PNA (ssPNA) has flexible, folded structure, while in PNA–DNA duplex its structure is extended and well defined. Therefore, it is sensible to assume that disulfide group in the interior of PNA will be differently shielded by neighbouring functional groups in free PNA and in the PNA bound to its target. It is also expected that reactivity of charged reagents in cleavage of positively charged ssPNA and PNA in negatively charged PNA–DNA duplex will be different. The aim of this work was to find reducing agents, which provide the strongest inhibition effect of DNA on cleavage of disulfide–PNA and determine factors controlling reactivity of disulfide–PNA in its free and hybridized forms.

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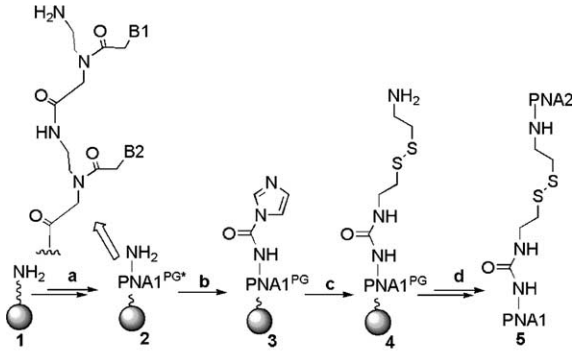
Disulfide modified PNAs **5a**–**c** were synthesized in accordance with Scheme 2. First, the PNA1 portion was prepared using solid phase synthesis. After activation of the terminal amino group of the PNA1 using CDI, a disulfide group was attached using cystamine dihydrochloride. Finally, the PNA2 portion was synthesized off the resulting terminal amino group and the desired product was cleaved from the polymeric support, deprotected using TFA/*m*-cresol and HPLC purified.

Internal modification in PNAs **5a**–**c** adds nine atoms to the PNA backbone. Providing that these PNAs form typical duplexes with their complementary DNAs, the modification should adopt a loop like structure. To test whether these PNAs bind DNAs sequence specifically UV-melting experiments were conducted (Table 1).



Scheme 2. Synthesis of internally modified PNA: (a) PNA synthesis, (b) 1. CDI, DMSO, (c) $(H_2NCH_2CH_2S)_2$, DIEA, DMSO, (d) 1. PNA synthesis, 2. TFA, *m*-cresol; (*) PG—protecting groups: Bhoc for nucleobases and Boc for lysine side chain NH_2, mismatched bases are underlined and shown bold, bulges are underlined and shown italic. B1 and B2—nucleobases.

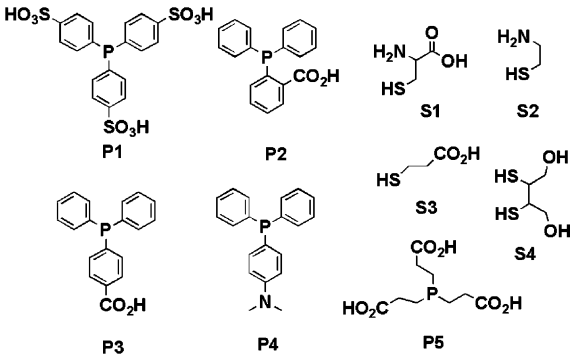
Table 1. Melting points of PNA–DNA duplexes

Entry	PNA:DNA[a]	Buffer[b]	T_m (°C)
1	**5a**:**6**	Water	39.2 ± 0.2
2	**5a**:**6a**	Water	28.2 ± 1.2
3	**5a**:**6b**	Water	23.0 ± 1.0
4	**5a**:**6c**	Water	20.4 ± 0.8
5	**5a**:**6**	20% CH_3CN in water	28.9 ± 0.2
6	**5a**:**6b**	20% CH_3CN in water	20.7 ± 0.3
7	**5a**:**6c**	20% CH_3CN in water	18.6 ± 0.9
8	**5a**:**6d**	Water	34.6 ± 0.4
9	**5a**:**6e**	Water	34.9 ± 1.5
10	**5b**:**7**	Water	66.3 ± 1.1
11	**5b**:**7**	20% CH_3CN in water	56.5 ± 1.8
12	**5c**:**8**	Water	62.1 ± 0.4
13	**5c**:**8**	20% CH_3CN in water	52.6 ± 0.1

[a] [PNA] = [DNA] = 2 μM.
[b] MOPS 1 mM, pH 7, NaCl 50 mM.

Another important question was whether both disulfide linked PNA parts bind complementary DNA. Indeed, the melting temperature of **5a**:**6** duplex is substantially higher than those of the **5a** duplexes with DNAs having single mismatches positioned on opposite sites of the internal modification (**6a**,**b**) or two mismatches **6c** (Table 1, entries 2–4). This indicates that both disulfide linked PNA parts in **5a** are bound to the complementary DNA (**6**). Dilute aqueous solutions of single stranded disulfide modified PNAs (PNA 1–10 μM, pH 7, MOPS buffer 1 mM) are not stable for a long time due to PNA absorption on walls of Eppendorf tubes or precipitation. Therefore, aqueous solutions containing 20% CH_3CN were used in all kinetics experiments. In this mixed solvent **5a**:**6** is less stable than in pure water, which is reflected in a T_m decrease, $\Delta T_m = 10.2$ °C. However, with these conditions, binding of **5a** to DNA is sequence specific as well (Table 1, entries 5–7). In analogous duplexes of the PNAs with DNAs containing bulge nucleotides opposite to the disulfide modification (+6 atoms per bulge in the DNA backbone), the loop in the PNA structure is expected to be more stretched. These distortions may explain at least in part destabilization of **5a**:**6d** and **5a**:**6e** in comparison with **5a**:**6** (entries 8 and 9). In all further experiments we have used more stable duplexes of PNA with DNAs containing no bulges.

To find conditions, with which the PNA cleavage reaction would be strongly affected by complementary DNA, a variety of reducing reagents were tested. We have selected phosphines (**P1**–**P5**) and thiols (**S1**–**S4**) (Scheme 3), which have different charges and sizes (Table 2). Representative HPLC's of mixtures of **P1** and **5a** with and without DNA are shown in Figure 1. As expected two shorter PNA fragments are formed in the result of **5a** cleavage. Assignment of the peaks in HPLC has been accomplished based on MALDI-TOF MS. In the presence of complementary DNA **6** and DNA **6b**, having a single mismatched nucleobase, traces of cleavage products are observed 5 h after addition of **P1** to **5a**:**6** (Fig. 1, trace 1). In contrast, in the presence of DNA **6c** having two mismatched nucleobases and in the absence of any DNA, cleavage of PNA **5a** is more substantial (Fig. 1, traces 3 and 4 correspondingly). The inhibition effect of DNA is strongly dependent upon its concentration. For example, in the presence of 3 equiv complementary DNA cleavage of **5a** by **P1** is practically



Scheme 3. Tested cleavers of disulfide modified PNA.

Table 2. Cleavage of disulfide–PNA by different phosphines and thiols

PNA:DNA	Time (min)	Cleaver (equiv)[a]	PNA cleavage %		SF[b]
			− DNA	+ DNA	
5a:**6**	300	**P1**, 10^2	88.1	3.7	23.8
5a:**6**[c]	300	**P1**, 10^2	97.6	21.0	4.6
5a:**6**	30	**P2**, 10^2	69.1	43.7	1.6
5a:**6**	30	**P3**, 10^2	92.9	88.6	1.0
5a:**6**	0.5	**P4**, 10^2	19.5	23.0	0.9
5a:**6**	2	**P5**, 10^2	100	70.7	>1[d]
5a:**6b**	2	**P5**, 10^2	100	98.6	⩾1[d]
5a:**6**	10	**S1**, 10^3	16.2	28.4	0.6
5a:**6**	2	**S2**, 10^3	86.8	56.1	1.5
5a:**6**	30	**S3**, 10^3	52.3	78.9	0.7
5a:**6**	30	**S4**, 10^3	77.2	7.6	10.2
5b:**7**	20	**P5**, 10^2	31.2	1.8	17.3
5b:**7a**	20	**P5**, 10^2	31.2	4.2	7.4
5b:**7b**	20	**P5**, 10^2	31.2	10.8	2.9
5b:**7**	300	**P1**, 10^2	91.1	9.0	10.1
5c:**8**	20	**P5**, 10^2	97.6	1.7	57.1
5c:**8a**	20	**P5**, 10^2	97.6	3.8	25.7
5c:**8b**	20	**P5**, 10^2	97.6	4.4	22.2
5c:**8a**	300	**P1**, 10^2	95.2	34.6	2.8

[a] See Scheme 3 for structural formulae of **P1**–**P5**, **S1**–**S4**.
[b] SF is a selectivity factor: ratio of amounts of cleaved PNA in the absence and in the presence of complementary DNA.
[c] With NaCl 0.5 M.
[d] Selectivity factor cannot be accurately calculated since the reaction in the absence of DNA has come to completion; [PNA] = 2 μM, [DNA] = 6 μM (for PNA 1) and 2 μM (for other PNAs), MOPS 1 mM, pH 7, 20% CH_3CN in water.

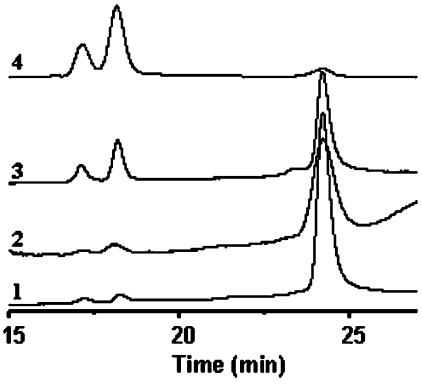


Figure 1. HPLC traces of mixtures of PNA **5a** (2 μM) with 3 equiv complementary DNA, **6** (trace 1), mismatch DNAs, **6b** (trace 2), **6c** (trace 3) and without any DNA (trace 4) obtained 5 h after addition of **P1** (200 μM). Buffer: MOPS 1 mM, pH 7 in CH_3CN/H_2O (1/4, v/v). PNA **5a** is eluted at 24.5 min, its cleavage products at 18.2 and 17.1 min.

fully suppressed (Supplementary data). However, at **[5a]**/**[6]** = 1 one observes considerable **5a** cleavage, which can be explained by incomplete formation of the PNA–DNA duplex (T_m = 29.0 ± 1.1 °C for **5a**:**6**). Therefore, we have used 3 equiv DNA for further cleavage experiments with **5a** to suppress duplex dissociation. Other synthesized disulfide–PNAs (**5b**,**c**) are fully bound by their complementary DNAs (Table 1, entries 10–13). Strong DNA inhibitory effects on reductive cleavage of these PNAs are observed already at equimolar DNA concentrations (Table 2). The ratio of the fractions of cleaved PNA at a specific time in the presence and absence of DNA is defined as the selectivity factor, **SF**. It roughly shows the difference in the reactivity of PNAs in bound and unbound forms, because, since we use considerable access of the reducing agent, the cleavage reaction is expected to be of pseudo-zero order relative to the PNA or PNA–DNA duplex (Table 2). All thiols, except dithiothreitol (**S4**, **SF** = 10.2), cleave PNA **5a** practically independently from the presence of complementary DNA (**SF** = 0.6–1.5) and they are less active in disulfide reduction than phosphines. The negatively charged bulky phosphine **P1** shows good selectivity in the PNA **5a** cleavage: **SF** = 23.8, while another poly-charged phosphine **P5** cleaves the PNA so quickly that the **SF** could not be determined at the concentration used for other phosphines (Table 2). Other studied phosphines (**P2**–**P4**) cleave the disulfide–PNA unselectively. The selective cleavage of the PNA by **P1** has been reproduced qualitatively for two other disulfide–PNA: **5b** and **5c** (Table 2). Inhibition by DNA of the PNA cleavage by **P1** is dependent from PNA sequence, which is reflected in a variation of the **SF** between 2.8 and 23.8. Cleavage by **P5** is quicker and more sensitive towards the PNA hybridization state than by other reagents (**SF** = 17.3 for **5b** and 57.1 for **5c**) and is sensitive to mismatches, which is reflected in the decreasing of **SF** by a factor of 2.2–6.0 for mismatch containing PNA–DNA duplex (Table 2). Accurate rates of **5b** cleavage by **P5** were determined in the presence and in the absence of complementary DNA **7** (Fig. 2).

The rates of **5b** cleavage are practically constant over the first 30 min (>50% disulfide cleavage) indicating a pseudo-zero order for this reaction. This confirms the validity of the previously introduced selectivity factors (**SF**) for a quick screening of ratios of reactivity of disulfide–PNAs in the reductive cleavage reaction in the presence and absence of complementary DNAs.

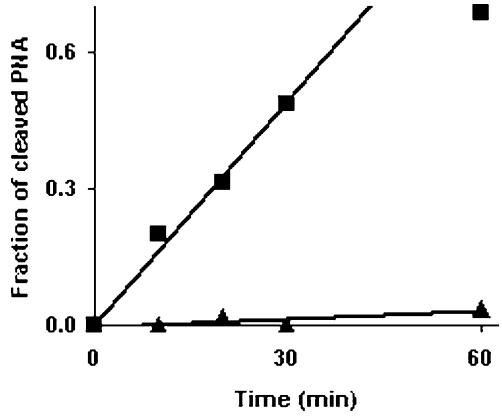


Figure 2. Cleavage of PNA **5b** by phosphine **P5** in the presence (▲) and absence (■) of complementary DNA **7**; MOPS 1 mM, pH 7, [PNA] = [DNA] = 8 μM, [**P5**] = 800 μM, CH_3CN/H_2O (1/4, v/v).

Several effects may contribute to the hybridization dependent cleavage of disulfide–PNAs. Firstly, specific recognition of the substrate by the reagent can play a role. Though, many enzymes have substrate specific binding pockets, the size of the reagent (phosphine or thiol) in our chemical model is too small to form such a pocket for the binding of PNA. Secondly, the disulfide in a well-defined rigid structure of the PNA–DNA duplex is less accessible for interaction with cleavers than in the unstructured flexible ssPNA.[4] One can expect that sterically demanding reagents will preferably cleave ssPNA. This mechanism is not in agreement with the fact that between similarly (−3)-charged triphenylphosphine—**P1** and trialkylphosphine—**P5**, the higher selectivity has been observed for the sterically less demanding trialkylphosphine. Thirdly, since ssPNA and the PNA–DNA duplex are differently charged, the electrostatic interaction between the substrate and the cleaver may be the decisive factor determining the selectivity of ssPNA cleavage. This is corroborated by the majority of our experimental data. In particular, singly negatively charged **P2**, **P3** and **S3**, as well as zwitter-ionic **S1** and positively charged **P4** and **S2** show no or little selectivity, whereas (−3)-charged phosphines **P1** and **P5**, which are expected to be strongly repulsed by negatively charged PNA–DNA duplexes, cleave efficiently only ssPNA.

The method of DNA sequence analysis based on selective binding of cationic poly(fluorenephenylene) derivatives with PNA–DNA duplexes has recently been reported.[6] The difference in affinity of the polymeric dye to positively charged ssPNA and negatively charged PNA–DNA is also defined by electrostatic interactions. Dithiothreitol—**S4** cleaves ssPNA quicker than PNA–DNA duplexes (**SF** = 10.2). Since **S4** is neutral, there is no substantial difference in the electrostatic interactions within pairs of reagent/ssPNA and reagent/PNA–DNA duplex. One can speculate that selectivity may be explained by steric reasons in this case, though it is unclear why **S4** does not follow the trend found for 8 other tested disulfide cleavers. The DNA inhibition effect in **5a** cleavage by triphenylphosphine—**P1** is reduced by a factor of 5.7 when the reaction is conducted in the presence of NaCl, 0.5 M, while salt has practically no effect on the cleavage of ssPNA (Table 2, entry 2). This fact also confirms the importance of electrostatic interactions in the studied cleavage. In the discovered chemical reaction as well as in DNA cleavage by unspecific nucleases single stranded substrates are more reactive than hybridized ones. Both our method and nuclease based methods allow discrimination between DNAs, which differ from one another by only one single base (Fig. 1, Table 2). Tris-(carboxyethyl)-phosphine, **P5** cleavages ss-disulfide–PNA substrates most selectively among tested reducing agents (cleavage rate of ssPNA/cleavage rate of PNA–DNA = 33). It should be noted that this simple chemical system is more selective than typical unspecific nucleases. In particular, phosphodiesterase I (EC 3.1.4.1) hydrolyzes ssDNA substrates five times quicker than dsDNA substrates[7] and unspecific endonucleases have comparable substrate selectivity.[8]

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Supplementary data

Description of synthesis of PNAs **5a**–**c**, conditions of UV-melting experiments, as well as conditions of reductive cleavage reaction. Dependence of PNA **5a** cleavage from presence of complementary DNA **6** and mismatched DNA **6c**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2004.11.066.

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