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Journal of Chromatography A, 922 (2001) 177–185

JOURNAL OF CHROMATOGRAPHY A

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# Recognition and strand displacement of DNA oligonucleotides by peptide nucleic acids (PNAs) High-performance ion-exchange chromatographic analysis

Francesca Lesignoli, Andrea Germini, Roberto Corradini*, Stefano Sforza, Gianni Galaverna, Arnaldo Dossena, Rosangela Marchelli

*Dipartimento di Chimica Organica e Industriale, Università di Parma, Parco Area delle Scienze 17/A, I-43100 Parma, Italy*

Received 8 January 2001; received in revised form 9 April 2001; accepted 11 April 2001

**Abstract**

Peptide nucleic acids (PNAs) are oligonucleotide mimics containing a pseudopeptide chain, which are able to bind complementary DNA tracts with high affinity and selectivity. Two mixed-sequence PNA undecamers (**1** and **2**) were synthesized and their double-stranded adducts with the complementary oligonucleotides (**3** and **4**) were revealed by the appearance of the corresponding peak in anion-exchange HPLC. A DEAE column was used and elution was performed with aqueous Tris buffer (pH 8) and an ionic strength gradient (0–0.5 *M* NaCl). The same effect was not observed with non-complementary oligonucleotides. The stability of the PNA–DNA adducts under the conditions used in the chromatographic system was studied as a function of temperature. Furthermore, in competition experiments double-stranded oligonucleotides were challenged by a PNA complementary to one strand: the formation of the PNA–DNA hybrid and the displacement of the non-complementary strand were observed with high specificity. The results suggest a possible use of ion-exchange HPLC for studying PNA–DNA interactions, and indicate the efficiency of PNA probes in the chromatographic analysis of DNA. 

*Keywords:* Ion exchange HPLC; Ionic strength gradient; Gradient elution; DNA recognition; Oligonucleotides; Peptide nucleic acids; Nucleic acids

## 1. Introduction

In the last years, there has been an enormous effort in providing new methods of detection and analysis of gene sequences, on account of the increasing impact of biotechnology on every day life. The most widely used methods for detecting specific DNA tracts of known sequences (such as those of genetically modified organisms) are based on hybridisation via Watson–Crick base pairing with complementary oligonucleotides, which can be used as probes (e.g., in situ hybridization assays) or as primers in polymerase chain reactions (PCR).

Peptide nucleic acids (PNAs) are oligonucleotide mimics in which the sugar-phosphate backbone has been replaced by a pseudopeptide chain of *N*-aminoethylglycine monomers (Fig. 1) [1]. PNAs were shown to bind to complementary DNA or RNA

*Corresponding author. Tel.: +39-521-905-410; fax: +39-521-905-410.

*E-mail address:* corradin@unipr.it (R. Corradini).

0021-9673/01/$ – see front matter 
PII: S0021-9673(01)00877-9

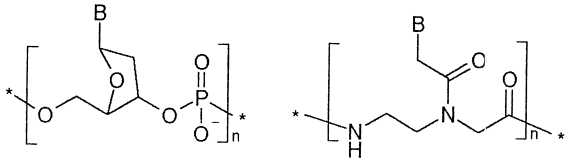

Fig. 1. DNA and PNA molecular structures.

sequences with high affinity and specificity [2,3]. On account of these properties, they are widely used in molecular biology and biotechnology, as tools in genetic diagnostics, for specific regulation of gene expression, and are currently investigated as potential antiviral and anticancer drugs [4].

In diagnostics, PNAs can turn out to be very useful, being able to form more stable hybrids than oligonucleotides with single- and, in some cases, double-stranded DNA. Unlike oligonucleotide probes, even short PNAs are expected to be efficient in hybridizing to target DNA [5]. Furthermore, the stability of PNA–DNA hybrids is greatly affected by the presence of a single base mismatch [6]. In addition, PNAs are expected to bind with high efficiency to single-stranded PCR products, independently from the secondary structure of the target DNA.

These properties have been used for detecting specific gene sequences in advanced diagnostic methods [7], by means of PCR clamping [8], real-time PCR [9], capillary electrophoresis [10,11], matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) [12], electrochemical biosensors [13–15], quartz crystal microbalance (QCM) [16], and microarrays [17]. Surface-plasmon resonance (BIAcore) biosensors have been used for the study of the hybridization kinetics of PNA–DNA duplexes [18] and have recently been proposed as an analytical method for the analysis of PCR products [19]. Single-molecule detection of transgenic DNA has also been performed by means of PNA probes and double wavelength fluorescence analysis [20].

In spite of the enormous effort to find new and sophisticated analytical methods for the application of PNA in diagnostics, there are no reports concerning the possible use of these compounds in HPLC for the detection of specific gene sequences. This technique has insofar only been used for the analysis and purification of PNAs after synthesis [21].

Synthetic oligonucleotides are routinely separated and purified by HPLC using ion-exchange [22] or reversed-phase columns [23–25], the latter technique being used for modified or tritylated oligonucleotides [26]. Oligonucleotides, as well as PCR fragments, can be easily identified by coupling HPLC with electrospray mass spectrometry [27]. The separation of double-stranded DNA (dsDNA) can be performed using ion-pair reversed-phase [28], or anion-exchange techniques [29]. The latter allows to detect either single-stranded (ss) [30] or double-stranded (ds) oligonucleotides [31] using non-denaturing eluents, and have been used for the analysis of PCR amplification fragments from natural samples [32–34].

On these accounts, we propose the use of ion-exchange HPLC for the detection of PNA–DNA adducts. In the present paper, the results obtained for the analysis of single- or double-stranded oligonucleotides using complementary PNA probes are described. The effects of the operating conditions on the stability of the adducts have also been studied and compared with data obtained in solution.

## 2. Experimental

### *2.1. Materials and reagents*

PNA monomers were from Perseptive Biosystems (Foster City, CA, USA), (4-methylbenzhydryl)amine (MBHA) resin was from Novabiochem (Calbiochem-Novabiochem, Laufeldingen, Switzerland). *O*-(1*H*-7-azabenzotriazolyl) -*N*,*N*,*N*′,*N*′- tetramethyluronium hexafluorophosphate (HATU), *N*,*N*-diisopropylethylamine (DIEA) and thioanisole were from Aldrich (Steinheim, Germany). *N*-Methylpyrrolidone (NMP), trifluoromethanesulfonic acid (TFMSA), trifluoroacetic acid (TFA) and *m*-cresol were from Fluka (Buchs, Switzerland).

All solvents used for HPLC were of chromatographic grade. Doubly distilled water was produced by a Millipore Alpha-Q purification module (Millipore, Bedford, MA, USA).

Oligonucleotides **3-6** (guaranteed oligos grade)

were purchased from Genset (Paris, France), and were used without further purification.

### 2.2. Synthesis of PNA*1* and *2*

The PNA **1** and **2** were synthesized manually, according to literature procedures [35–38]. One hundred mg of MBHA resin (0.8 meq/g) were downloaded overnight with the first PNA monomer (0.010 mmol) in the presence of HATU (7.3 mg, 0.019 mmol) as coupling agent, DIEA (8 μl) as base, and *N*-methylpyrrolidone (NMP, 250 μl) as solvent. Capping after first monomer loading was carried out with an acetic anhydride–pyridine–NMP (1:2:2, v/v/v) mixture.

The remaining PNA part was then synthesized using the following conditions: (i) *deprotection*: trifluoroacetic acid–*m*-cresol (95:5, v/v) mixture; (ii) *coupling*: with Boc-PNA monomers (0.072 mmol, in 280 μl of NMP), DIEA (21.8 μl, 0.150 mmol in 228.2 μl of pyridine), and HATU (24.3 mg, 0.064 mmol in 250 μl of NMP) with preactivation for 2 min; (iii) *capping* with an acetic anhydride–pyridine–NMP 1:25:25 (v/v/v) mixture. Cleavage of the oligomer from the resin after the last step was carried out by treatment with a TFA-TFMSA thioanisole–*m*-cresol (6:2:1:1) mixture. The solvent was partially evaporated and the product was precipitated with diethyl ether. The crude product was purified by reversed-phase HPLC using a Vydac $C_{18}$ peptide (5 μm, 20 cm×4.5 mm) column and the following binary gradient (min, %A): 0, 100%; 3, 98%; 8, 90%; 25, 86%; (flow-rate: 1 ml/min). Eluent A: water–TFA (1000:1); eluent B: acetonitrile–TFA (1000:1). Detector UV (260 nm).

The products were identified by mass spectrometry.

PNA (**1**): H-AGAGTCAGCTT-$NH_2$. Yield: 68%. MALDI-TOF-MS: mass calculated from the sequence for $MH^+$: 2993.4, found: 2993.

PNA (**2**): H-AAGTCGTCGCT-$NH_2$. Yield: 90%. MALDI-TOF-MS: mass calculated from the sequence for $MH^+$: 3017.4, found: 3016.

### 2.3. Melting temperature measurements

Stock solutions of PNA and DNA were prepared in doubly distilled water and their concentration was measured by absorbance using the following $\varepsilon_{260}$ ($M^{-1}$ $cm^{-1}$) for the nucleobases: T 8800, C 7300, A 10 400, G 11 700.

All hybrid samples reported were first incubated at 90°C for 5 min, then slowly cooled at room temperature. All hybridization experiments were carried out in a 10 m*M* phosphate buffer, 100 m*M* NaCl, 0.1 m*M* EDTA, pH 7. Melting curves were recorded on a Perkin-Elmer λ BIO 20 spectrophotometer, equipped with a PTP 6 Peltier temperature programmer and a cell changer (Perkin-Elmer, Norwalk, CT, USA). The samples were heated (1°C/min, 5 points/min) and the UV signal variation at 260 nm was recorded. Melting temperatures were taken as the maxima of the first derivative of the melting curves.

### 2.4. HPLC measurements

All purifications and experiments were carried out on an Alliance Waters 2690 Separation Module HPLC system, equipped with a temperature controller and a dual-wavelength absorbance detector Waters 2487 (Waters, Milford, MA, USA). Anion-exchange chromatographic measurements were carried out using a Vydac 301 VHP575 column (50×7.5 mm) (Vydac, Hesperia, CA, USA), which has diethylaminoethyl (DEAE) functional groups linked to a polystyrene–divinylbenzene (PS–DVB) solid support. A binary linear gradient was used for elution from 100% A (0.05 *M* Tris–HCl in water, pH 8) to 100% B (0.05 *M* Tris–HCl, 0.5 *M* NaCl in water, pH 8) in 60 min, with flow-rate 1 ml/min and UV detection at 260 nm. The dead time ($t_0$) was calculated by injection of methanol (detection at 220 nm).

Single- and double-stranded oligonucleotide and PNA samples were prepared in 10 m*M* phosphate, 0.1 *M* NaCl, 0.1 m*M* EDTA buffer (pH 7), incubated at 90°C for 3 min, and slowly cooled to room temperature before injection in the HPLC. Sample concentration (in each strand) was 5 μ*M*, and injection volume was 30 μl (total amount: 150 pmol of each strand) in all experiments.

For experiments carried out at different temperatures, the samples were preincubated as described above and kept at the working temperature in a

thermostat before injection, in order to achieve temperature homogeneity.

For strand displacement experiments, the DNA–DNA duplexes were prepared and incubated as reported above and injected in HPLC, then a 2-fold excess of PNA (10 μ*M* final concentration) was added, and the samples were either incubated at 90°C and slowly cooled to the desired temperature or directly injected without incubation.

## 3. Results and discussion

### *3.1. PNA synthesis*

The PNA oligomers **1** and **2** (Table 1), were synthesized by solid-phase synthesis on a MBHA resin, using HATU–DIEA coupling. The length of PNA (undecamer) was chosen in order to provide sufficient stability to the DNA–PNA duplexes. The products were obtained in good yields (68–90%) and were purified by RP-HPLC. The molecular mass was determined by MALDI-TOF-MS analysis, as described in the Section 2.

### *3.2. Retention times of single-stranded oligonucleotides and PNAs*

The retention times of PNA **1** and **2** and of the complementary (**3** and **4**) or homologous (**5** and **6)** oligonucleotides individually injected were measured by using an ion-exchange DEAE column (Vydac 301 VHP575) and an ionic strength gradient (0–0.5 *M* NaCl). As expected, the oligonucleotides were eluted with high retention time, due to the electrostatic interactions of the phosphate groups with the cationic stationary phase, while the PNA oligomers, which at this pH are positively charged (at the terminal amino group) were practically not retained by the column (Table 1). The oligonucleotides, though bearing the same number of charges, displayed sequence-dependent retention times [39], indicating additional hydrophobic interactions with the stationary-phase, which has a PS–DVB solid support.

### *3.3. Detection of DNA–DNA and PNA–DNA adducts*

In order to evaluate if the HPLC technique could be useful for detecting double-stranded adducts, we incubated complementary DNA and PNA strands

Table 1
HPLC retention times ($t_R$) and capacity factors ($k$) for single stranded PNAs **1**–**2** and nucleotides **3–6**, and for double stranded PNA–DNA and DNA–DNA adducts at room temperature[a]

| Compound/adduct | Sequence | $t_R$ (min) | $k$ |
|---|---|---|---|
| PNA **1** | H-AGAGTCAGCTT-$NH_2$ | 1.33 | 0 |
| PNA **2** | H-AAGTCGTCGCT-$NH_2$ | 1.35 | 0.01 |
| DNA **3** | 5′-AAGCTGACTCT-3′ | 17.50 | 12.15 |
| DNA **4** | 5′-AGCGACGACTT-3′ | 18.10 | 12.61 |
| DNA **5** | 5′-AGAGTCAGCTT-3′ | 15.50 | 10.65 |
| DNA **6** | 5′-AAGTCGTCGCT-3′ | 16.80 | 11.63 |
| DNA **3**–DNA **5** | 3′-TCTCAGTCGAA-5′<br>5′-AGAGTCAGCTT-3′ | 19.03 | 13.31 |
| DNA **4**–DNA **6** | 3′-TTCAGCAGCGA-5′<br>5′-AAGTCGTCGCT-3′ | 19.25 | 13.47 |
| PNA **1**–DNA **3** | H-AGAGTCAGCTT-$NH_2$<br>3′-TCTCAGTCGAA-5′ | 14.47 | 9.88 |
| PNA **2**–DNA **4** | H-AAGTCGTCGCT-$NH_2$<br>3′-TTCAGCAGCGA-5′ | 14.28 | 9.73 |

[a] Chromatographic conditions: Vydac 301 VHP575 column (50×7.5 mm) and UV detection (260 nm). Binary linear gradient from 100% A (0.05 *M* Tris–HCl in water, pH 8) to 100% B (0.05 *M* Tris–HCl, 0.5 *M* NaCl in water, pH 8) in 60 min flow-rate 1 ml/min. $t_0$=1.33 min.

(**1:3**, **2:4**, **3**:**5** and **4:6**) at 90°C in a phosphate buffer (pH 7.0, 0.1 *M* NaCl) for 2 min, and slowly cooling at room temperature for 30 min. The resulting solutions were then injected into the HPLC system. The duplex formed by two complementary DNA strands (**3**:**5** or **4:6**) gave rise to a peak with retention time longer than those observed with the two single-stranded oligonucleotides. In contrast, the PNA–DNA duplexes (**1:3**, **2:4**) showed retention times intermediate between those of ssDNA and of PNA (Table 1). Therefore, both types of adducts (PNA–DNA and DNA–DNA) are stable under the analytical conditions used, and give interactions with the HPLC column which do not produce duplex denaturation. Examples of chromatograms of PNA, ssDNA, dsDNA and PNA–DNA mixtures are reported in Fig. 2. By injecting a mixture of a PNA with the non-complementary DNA sequence (**1** and **4** or **2** and **3**), the predominant peaks were identified as ssDNA and PNA, thus confirming that the PNA–DNA interaction was sequence-specific. However, a small peak at a retention time similar to that of PNA–DNA adduct was observed, probably due to a weak partial hybridization.

It is worth noticing that the PNA–DNA adducts are easily detectable, since their retention times differ noticeably from those of single- and double-stranded DNA, thus suggesting a possible use of PNA probes for the detection of specific DNA tracts.

### *3.4. Temperature dependence of PNA–DNA hybrid formation*

The stability of the PNA–DNA adducts was evaluated by means of UV melting curves in aqueous solution (10 m*M* phosphate buffer, pH 7.0, 0.1 *M*

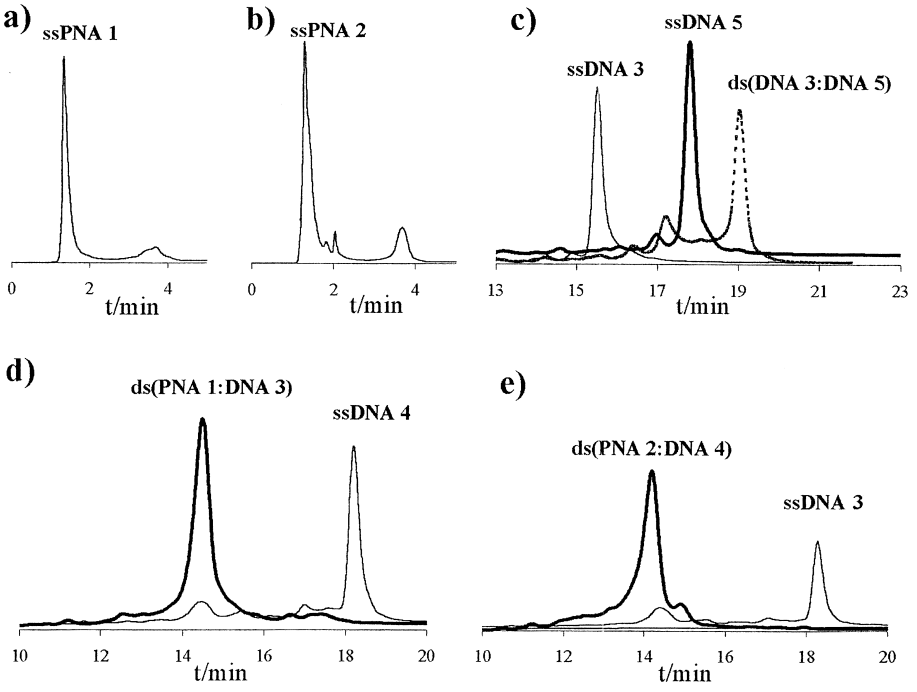


Fig. 2. Ion-exchange chromatograms at room temperature of: (a) PNA **1**; (b) PNA **2**; (c) DNA **3**, (thin solid line) DNA **5** (thick solid line), and DNA **3**–DNA **5** mixture (thin broken line); (d) PNA **1**–DNA **3** (thick line) and PNA **1**–DNA **4** (thin line) mixtures; (e) PNA **2**–DNA **4** (thick line) and PNA **2**–DNA **3** (thin line) mixtures. Conditions for all chromatograms were as follows: Vydac 301 VHP575 column (50×7.5 mm) and UV detection (260 nm). A binary linear gradient was used for elution from 100% A (0.05 *M* Tris–HCl in water, pH 8) to 100% B (0.05 *M* Tris–HCl, 0.5 *M* NaCl in water, pH 8) in 60 min flow-rate 1 ml/min. A total of 150 pmol of each strand was injected in all cases.

NaCl, 0.1 m*M* EDTA); the PNA **1**:DNA **3** and PNA **2**:DNA **4** hybrids were found by this technique to have a melting temperature of 66 and 62°C, respectively. On the contrary, the melting profiles obtained for the PNA **1**–DNA **4** and PNA **2**–DNA **3** mixtures did not show definite transitions, confirming the selectivity of PNA–DNA interactions.

In order to prove the nature of the peaks attributed to PNA–DNA hybrids, and to study the temperature dependence of these under chromatographic conditions, we performed the HPLC analysis of a mixture of PNA **1** and DNA **3** at different temperatures. The results are summarized in Fig. 3.

At high temperature (55°C) only the two peaks assigned to single-stranded PNA and DNA were detected, while at 45°C a small peak of the PNA–DNA adduct started to be observed, becoming predominant at 40°C and completely formed at 35°C. Therefore, it is possible to visualize directly the thermal stability of PNA–DNA hybrids under HPLC conditions. The evaluation of the relative area of the PNA–DNA duplex peak as a function of temperature (Fig. 3b) shows the typical sigmoidal profile usually observed in UV melting experiments in solution. Similar results were obtained with the duplex PNA **2**–DNA **4**.

However, the temperatures at which the PNA–DNA duplexes are stable in HPLC are lower than those obtained by UV melting measurements, suggesting that the interaction with the stationary phase and the dynamic removal of the interacting strands induce a slight denaturation of these complexes.

### *3.5. Strand displacement*

In many diagnostic applications double-stranded rather than single-stranded DNA must be targeted. It has been reported that homopyrimidine PNAs are able to bind to a complementary sequence of dsDNA through formation of PNA–DNA–PNA triplexes, with displacement of the non-complementary strand [40]. However, strand displacement by mixed sequence PNAs is not a common mechanism since the formation of DNA–DNA duplexes competes and can be the favorite process at high ionic strength [41].

In order to verify if strand displacement could be

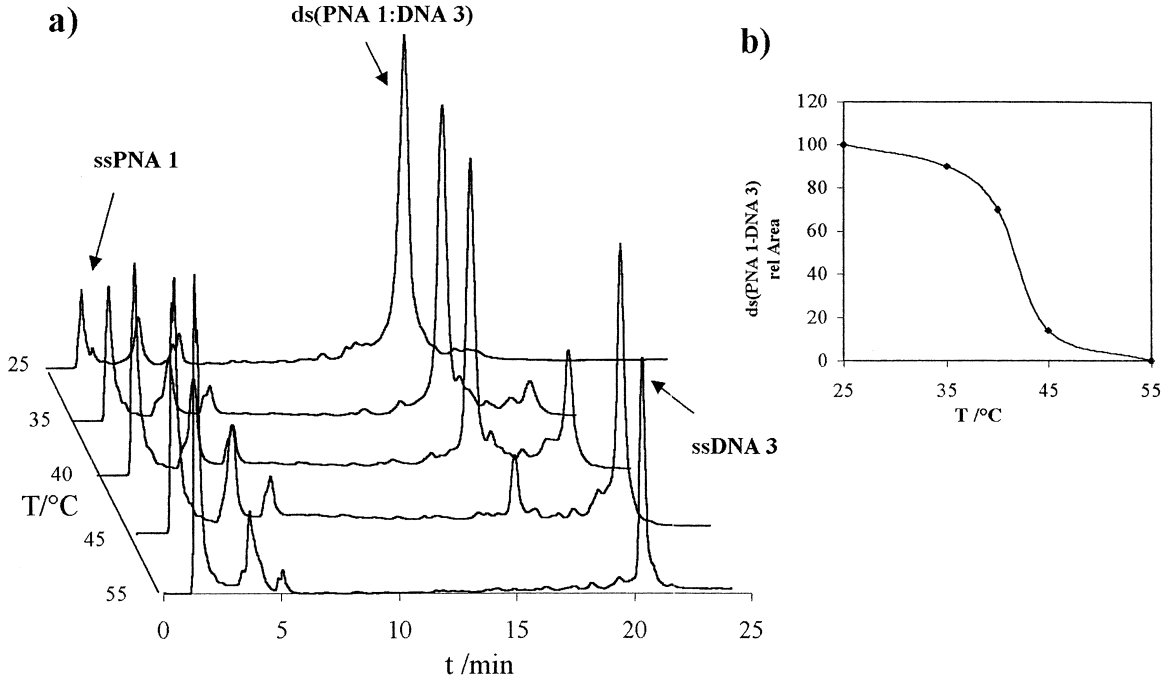


Fig. 3. Temperature dependence of chromatographic profiles for a 1:1 mixture of PNA **1** and DNA **3** ($c = 5$ μ*M*, injection volume: 30 μ*M*). All other conditions as in Fig. 2.

directly observed by a HPLC system using mixed-sequence PNAs, we prepared the hybrid between two complementary oligonucleotides (**3:5**) and then we added PNA **1**, which is complementary to DNA **3**, in a 2:1 ratio. The results are reported in Fig. 4.

By injecting the DNA **3**–DNA **5** mixture, we observed the presence of a peak corresponding to the duplex (Fig. 4d), suggesting that the dsDNA is stable under the present conditions (35°C, pH 8.0). However, when this solution was added of an excess (2:1) of PNA **1** (Fig. 4e), the DNA **3**–DNA **5** duplex disappeared giving rise to the formation of two peaks, the former corresponding to the PNA **1**–DNA **3** duplex, the latter to single stranded DNA **5**, according to the strand displacement process:

$$\text{ds(DNA } \mathbf{3}\text{–DNA } \mathbf{5}\text{)} + \text{ssPNA } \mathbf{1} \rightleftharpoons \text{ds(DNA } \mathbf{3}\text{–PNA } \mathbf{1}\text{)} + \text{ssDNA } \mathbf{5} \quad (1)$$

as confirmed by comparison with the corresponding chromatograms at the same temperature (compare Fig. 4d,e). The strand displacement turned out to be fast, since samples prepared at room temperature and those incubated at 90°C gave essentially the same results. Therefore, preincubation is not needed in this type of analysis.

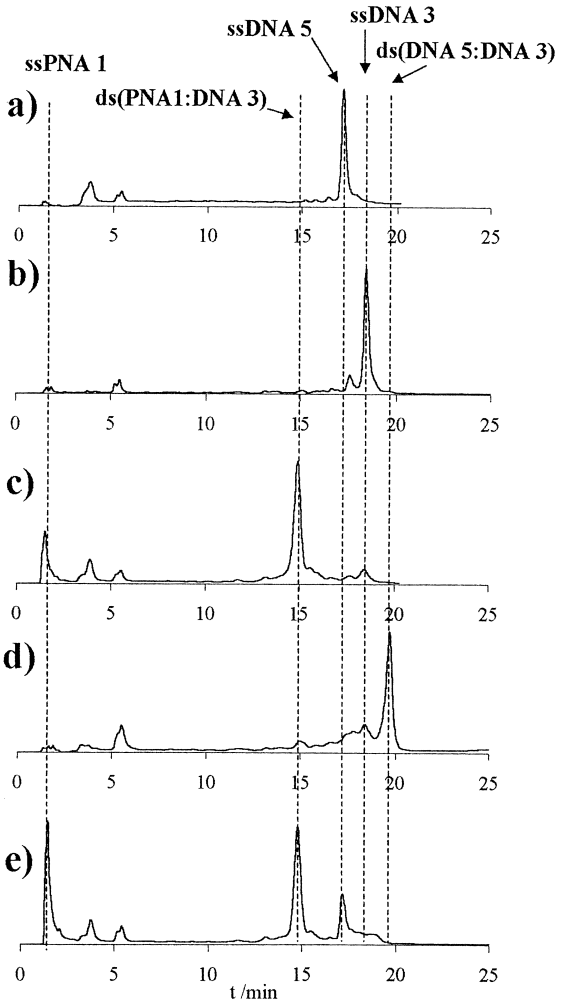


Fig. 4. Ion-exchange chromatograms at 35°C of: (a) DNA **5**; (b) DNA **3;** (c) PNA **1**–DNA **3**; (d) DNA **3**–DNA **5**; (e) PNA **1** added to solution (d). All other conditions as in Fig. 2.

The same type of experiment was performed by adding PNA **2** (which is complementary to DNA **4**) to a solution of the duplex DNA **4**–DNA **6** at 25°C (compare Fig. 5a,b).

It is worth noticing that the addition of non-complementary PNA **1** to the DNA **4**–DNA **6** duplex did not give rise to any PNA–DNA adduct (Fig. 5c), suggesting that the recognition of dsDNA is highly sequence-selective and interference due to partial hybridization (as those reported in Fig. 2 for ssDNA) is not relevant.

## 4. Conclusions

In recent years the need for sequence-specific DNA analysis has spread from the biomedical field to other areas, such as food and feed analysis, for which non-expensive, fast and easy to handle techniques would be preferred.

In the present paper we have demonstrated for the first time that ion-exchange HPLC can be used in combination with PNA technology for the sequence-specific recognition of oligonucleotides. The use of this widely diffused and easily accessible equipment is highly desirable in genetic analysis. The detection of PNA–DNA hybrids by UV absorption described in the present work was performed at a 150-pmol scale; work is in progress in our group for increasing the sensitivity and selectivity of detection, by means of fluorescent PNA probes.

Furthermore, we have demonstrated for the first time that HPLC can be used for directly monitoring the formation of PNA–DNA adducts, providing a

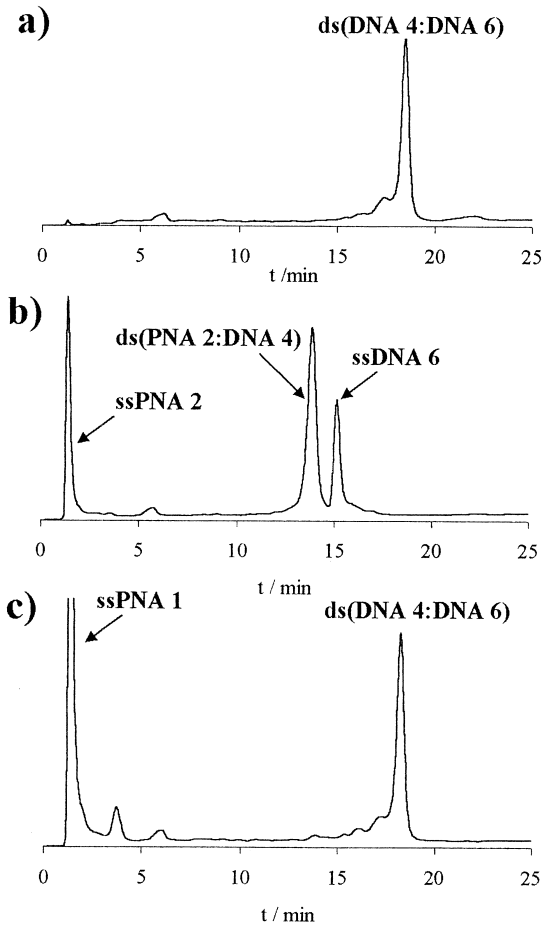


Fig. 5. Ion-exchange chromatograms at 25°C of: (a) DNA **4**:DNA **6**; (b) PNA **2** added to solution (a); (c) PNA **1** added to solution (a). All other conditions as in Fig. 2.

powerful and fast tool (complementary to ''gel-shift'' experiments) for evaluating the recognition properties of PNA and their analogs, which is fundamental for the design of new DNA binding compounds.

Finally, we have demonstrated that strand displacement of dsDNA can be detected by a simple chromatographic system, paving the way to HPLC methodologies targeted to genomic DNA or PCR amplification products. This method can also be useful for evaluating the conditions under which strand displacement, important in anti-gene strategy [42], can occur.

## Acknowledgements

This work was partially supported by a grant from CNR, Progetto Finalizzato Biotecnologie, and from MURST ''Application of PNAs, Rybozymes and Peptides for the Study of gene Expression and Development of new Diagnostic and Therapeutic Strategies''. We wish to thank Professor PietroTraldi and Dr. Roberta Seraglia for the MALDI-TOF-MS analysis of PNAs.

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