2792–2799 *Nucleic Acids Research, 1997, Vol. 25, No. 14*

Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays

Jan Weiler, Heinrich Gausepohl[1], Nicole Hauser, Ole N. Jensen[2] and Jörg D. Hoheisel*

Molecular-Genetic Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 506, D-69120 Heidelberg, Germany, [1]ABIMED Analysentechnik GmbH, Raiffeisenstraβe 3, D-40764 Langenfeld, Germany and [2]Protein and Peptide Group, European Molecular Biology Laboratory, Meyerhofstraβe 1, D-69117 Heidelberg, Germany

Received April 3, 1997; Revised and Accepted June 4, 1997

ABSTRACT

Arrays of up to some 1000 PNA oligomers of individual sequence were synthesised on polymer membranes using a robotic device originally designed for peptide synthesis. At ~96%, the stepwise synthesis efficiency was comparable to standard PNA synthesis procedures. Optionally, the individual, fully deprotected PNA oligomers could be removed from the support for further use, because an enzymatically cleavable but otherwise stable linker was used. Since PNA arrays could form powerful tools for hybridisation based DNA screening assays due to some favourable features of the PNA molecules, the hybridisation behaviour of DNA probes to PNA arrays was investigated for a precise understanding of PNA–DNA interactions on solid support. Hybridisation followed the Watson–Crick base pairing rules with higher duplex stabilities than on corresponding DNA oligonucleotide sensors. Both the affinity and specificity of DNA hybridisation to the PNA oligomers depended on the hybridisation conditions more than expected. Successful discrimination between hybridisation to full complementary PNA sequences and truncated or mismatched versions was possible at salt concentrations down to 10 mM Na^+ and below, although an increasing tendency to unspecific DNA binding and few strong mismatch hybridisation events were observed.

INTRODUCTION

In recent times, the number of possible applications of nucleic acid hybridisation techniques in molecular biology and medicine has grown immensely. Especially the stimulus by the fast increase in genetic information that becomes known and thus available for genetic analysis procedures has spurred technical developments in this field. The capacity to assay quickly very many samples makes this technology almost indispensable for a variety of applications (for a review see 1). Different methods for the creation of DNA arrays are being used, but the basic idea on how to perform analyses remains the same: hybridisation of an unknown DNA sample to an ordered array of immobilised DNA sensor molecules of known sequence produces a specific hybridisation pattern which can be analysed and compared to a given standard. The sensor molecules consist of synthetic oligonucleotides or longer, enzymatically generated DNA, specifically PCR products and isolated clone DNA. Array production is being done either by a transfer of individual DNAs onto a solid support (2–4) or, in the case of oligonucleotides, alternatively by an *in situ* synthesis (5–8).

Although there are many promising developments and a few already working systems, some problems remain. One important aspect is the sensitivity and selectivity of the binding of the assayed DNA molecules. With long sensor molecules (PCR products) the sensitivity is less of a problem, since the stability of the duplex formed with the target sequence is rather high. However, there is a pay-off in the form of reduced selectivity. Certain assays, most obviously DNA sequencing by hybridisation (SBH; for short reviews see 9,10), do require the use of oligonucleotides. Oligomers, though, have the inherent disadvantage of a relatively low duplex stability. An optimal sensor molecule should combine the specificity of DNA oligomers with a relatively high duplex stability.

Second, DNA sequences form stable duplexes only in the presence of salt which is needed to counteract the interstrand repulsion. Such conditions, however, also stabilise secondary and tertiary structures within a target molecule. Sequences might thereby not be accessible and be prevented from hybridisation to the gridded DNA (11).

The use of peptide nucleic acid (PNA) oligomers could circumvent the above problems. In this nucleotide analogue the sugar backbone has been replaced by *N*-(2-aminoethyl)-glycine units (Fig. 1) but the bases nevertheless form a specific duplex structure with complementary DNA sequences. Since its invention (12), PNA has been intensively studied, because of its potential as a gene-targeting drug or a biomolecular probe. The PNA–DNA duplexes that were analysed in solution exhibited a very high thermal stability due to the missing interstrand repulsion between the DNA phosphate groups and the uncharged peptide backbone of the PNA molecules. The stability is reported to increase at physiological conditions by ~1.5°C per base pair

*To whom correspondence should be addressed. Tel: +49 6221 424680; Fax: +49 6221 424682; Email: j.hoheisel@dkfz-heidelberg.de

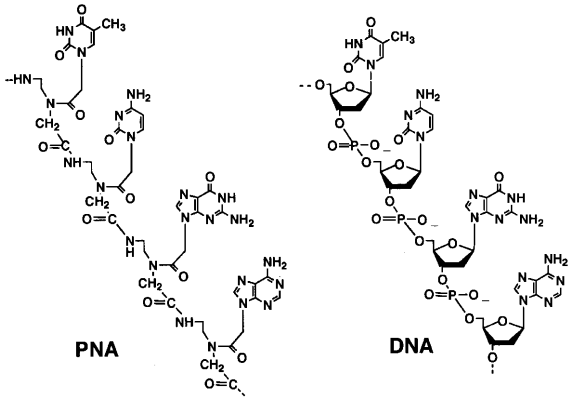


Figure 1. PNA consists of repeating units of *N*-(2-aminoethyl)-glycine linked by amide bonds. The nucleobases are attached to the backbone by ethylene carbonyl linkages.

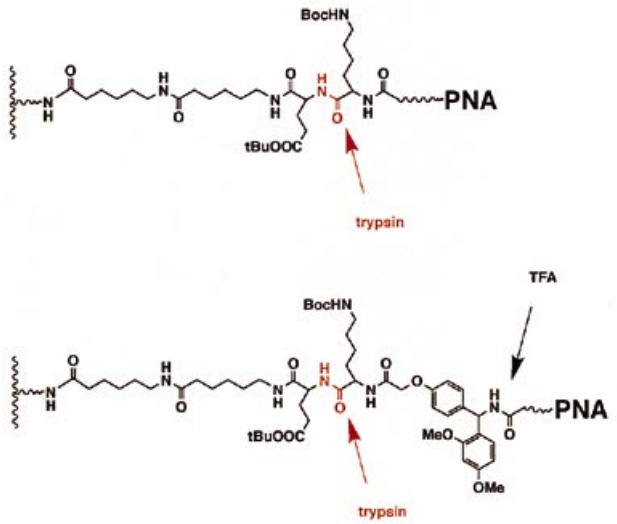


Figure 2. Linker chemistry used for the automated SPOT synthesis of PNA oligomers. The growing PNA chain is linked to the peptide spacer Glu[OtBu]-εAhx-εAhx via an enzymatically cleavable Glu–Lys handle and an optional acid-labile Rink-amide linker.

compared to that of the equivalent DNA–DNA hybrid (13). Furthermore, the base pair mismatch discrimination of PNA seems to be as good as or even superior to that of DNA (14).

Here, the synthesis of PNA arrays and their use in hybridisation assays is reported. For quality control, linkers were employed that allow the release of the intact PNA molecules after synthesis. Obviously, this mechanism could be utilised for a parallel synthesis of many different oligomers and the subsequent removal of individual sequences for other analyses, similar to a recent report on DNA oligonucleotide arrays (8). On the grids, studies on the interaction of PNA and DNA molecules of complementary sequence were carried out, with the PNA attached to solid support. The thorough understanding of the hybridisation under such conditions is not only instrumental for the use of PNA as sensor molecules in the oligomer array technology but could also be used to screen for sequences that are most suited for various applications, such as the antisense strategy.

MATERIALS AND METHODS

Chemicals

9-Fluorenylmethoxycarbonyl (Fmoc)-protected PNA monomers with the exocyclic amino groups of A, G and C being blocked with the benzhydryloxycarbonyl (Bhoc) group, 1-hydroxyazabenzotriazole (HOAt) and amino-functionalised membranes were obtained from PerSeptive Biosystems Inc. (Framingham, USA). Amino acid Fmoc-derivatives were from Novabiochem-Calbiochem (Läufelfingen, Switzerland). Solvents and reagents for peptide and PNA chemistry were from Fluka (Neu-Ulm, Germany), trypsin from Boehringer Mannheim (Germany). Unlabelled and 5′-digoxygenin (5′-DIG) labelled DNA oligonucleotides were obtained from Interactiva (Ulm, Germany). [γ-^{32}P]ATP (10 mCi/ml), T4 polynucleotide kinase and buffers for radioactive labelling of DNA oligomers at their 5′-position were purchased from Amersham and New England Biolabs, respectively. DIG labelled uridine-triphosphates and kits for their use in PCR amplification and subsequent fluorescence detection were from Boehringer Mannheim (Mannheim, Germany).

Linker attachment

In three synthesis cycles, the amino-functionalised membrane was derivatised with the peptide spacer glutamic acid-(γ-*tert*-butylester)-(ε-aminohexanoic acid)-(ε-aminohexanoic acid) (Glu[OtBu]-εAhx-εAhx; Fig. 2) using standard Fmoc-chemistry. The respective amino acid derivative was activated by the addition of 1.2 equivalent diisopropycarbodiimide (DIC) and 1 equivalent HOAt and used at a final concentration of 0.2 M in *N*-methyl-2-pyrrolidone (NMP) with no preactivation. For coupling, membranes were submerged in this solution for 15 min using a flat polypropylene container with lid. The membranes were then washed with dimethylformamide (DMF), and the Fmoc-groups were removed by a 5 min incubation in 20% piperidine in DMF. The membranes were again washed in DMF, rinsed with ethanol and dried in a stream of cold air.

Subsequent to the addition of the spacer, the membranes were mounted in the ASP 222 Automated SPOT Robot (15; ABIMED GmbH, Langenfeld, Germany) and a grid of the desired format was spotted using at each position 0.3 µl of activated Fmoc-lysine-(ε-*tert*-butyloxycarbonyl) (Fmoc-Lys[Boc]). After a reaction time of ~30 min, the membranes were treated with 5% acetic anhydride in dry NMP to cap all amino groups outside the spotted areas. The membranes were washed and deprotected as described above and the spots were visualised by treating the membrane with a solution of 0.01% bromophenol blue in DMF (16). Optionally, the acid cleavable Rink-amide linker (17) was added in another cycle (Fig. 2).

PNA array synthesis

In each cycle, 20 µmol of each Fmoc-protected PNA monomer were dissolved in 55 µl of 0.4 M DIC in NMP and 55 µl of 0.4 M HOAt in NMP to yield 125 µl of activated mixture. Reagent solutions were used after ~5–10 min preactivation, a time required for complete dissolution, centrifugation and instrument preparation.

Aliquots of 0.4 µl of the 0.16 M solution were deposited to the individual spots on the membranes using the ASP 222 Automated SPOT Robot. The complete synthesis cycle comprised: coupling (spotting of activated derivative followed by 20 min reaction time after placing material to the last spot); 5 min of acetylation in 5% acetic anhydride in DMF; five washes of 1 min each in 10 ml DMF; 5 min of deprotection with 20% piperidine in DMF; staining with 0.01% bromophenol blue in DMF; three rinses in 10 ml ethanol and subsequent drying. Usually, arrays of 384 (16 × 24) or 576 (18 × 32) spots were synthesised. The area of each spot was ~0.2 cm^2 with a final loading of ~10 nmol crude PNA compound. After completion of the synthesis, the PNA oligomers were deprotected by a 1 h incubation in a mixture of 90% trifluoroacetic acid (TFA), 5% water and 5% triethylsilane.

Release of PNA oligomers

Spots on the PNA array were visualised with bromophenol blue and pieces of the desired spots of were cut out. They were rinsed for 5 min in 33% acetonitrile/70 mM ammoniumbicarbonate and 5 min in 100 mM ammoniumbicarbonate to remove the bromophenol blue stain. To release the PNA oligomers from the solid support, 10 µl of 30 ng/µl bovine trypsin solution (Boehringer Mannheim, sequencing grade) in 50 mM ammoniumbicarbonate was added followed by an incubation at 37°C, cleaving the lysyl–glutamic acid bond. The digestion was terminated after 3 h by addition of neat formic acid to a final concentration of 10% acid. PNA molecules attached to the Rink-amide handle could alternatively be cleaved off by TFA during the PNA side chain deprotection. The crude PNA compound was extracted with 20% acetonitrile in water and analysed by mass spectrometry.

Matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry

MALDI matrix surfaces were prepared by the fast evaporation method using a solution of 4-hydroxy-α-cyanocinnamic acid and nitrocellulose in acetone/isopropanol (18). This matrix, which is optimal for peptide analysis, was chosen because PNA has a peptide amide backbone instead of a DNA phosphodiester backbone for which other types of matrices are preferable. An aliquot of 0.5 µl of PNA solution was deposited onto the matrix surface and allowed to dry at ambient temperature. The sample deposit was rinsed with 10 µl of 5% formic acid. Samples were mass analysed using a MALDI time-of-flight mass spectrometer (REFLEX II, Bruker Daltonics, Bremen, Germany) equipped with delayed ion extraction. Spectra were acquired as the sum of signals generated by irradiation of the sample deposit with 100–200 laser pulses. The resulting mass spectra were mass calibrated by using trypsin autodigestion peptides (m/z 2163.057, 2289.155) as internal standards. Monoisotopic molecular masses were determined.

Hybridisation of DNA oligonucleotides

DNA oligonucleotide probes were 5′-labelled with [γ-^{32}P]ATP as described earlier (8). After heat denaturation, hybridisation was in 10–20 ml of 60 mM sodium citrate, pH 7.5, 7.2% sodium *N*-lauroylsarcosine and NaCl with about ~1 Mc.p.m./ml of labelled probe (concentration = 2–5 pmol/ml); the Na^+ concentration, the temperature (0–90°C) and the duration of hybridisation were varied in different experiments. Subsequent to hybridisation, the membrane was washed for 30–60 min in the same buffer. In some cases, gradual washing at higher temperatures was done in order to obtain better discrimination. Exposure was by autoradiography, at room temperature or at –70°C using intensifying screens, and by a phosphorimager. Alternatively, hybridisation was carried out with fluorescently labelled probes. For indirect detection of enzymatically amplified fluorescence, 20 pmol of 5′-DIG labelled oligomers or DIG-dUTP labelled PCR fragments were hybridised to PNA membranes as described above. Fluorescence signals upon dephosphorylation of added AttoPhos™ substrate were obtained as described (19). Alternatively, direct detection was carried out via fluorescein or infrared dye labels. Data quantification was done by processing the hybridisation results with the BioImage HDG Analyser software, version 2.1, run on a SUN Sparc 5 station. Finally, probes were stripped off by incubating the membranes in boiling hybridisation buffer for several hours. Sometimes, membranes were also treated with 90% TFA in water at room temperature to remove very strongly bound molecules.

RESULTS

PNA synthesis on membrane supports

As opposed to DNA synthesis, which usually is based on phosphoramidite chemistry, the synthesis of PNA oligomer grids is greatly simplified by the fact that no complete absence of moisture is required. The peptide character of the compounds allows for the application of the SPOT method originally developed by Frank (15) for parallel oligopeptide synthesis. In a recent communication, we reported the suitability of the automated SPOT method for the generation of arrays of short PNA sequences (20).

Since the reliability of hybridisations to oligomer arrays depends strongly on the quality of the surface-bound oligomers (e.g., 21), it was essential for an accurate assessment of the performance of the PNA membranes to check the quality of the PNA *in situ* synthesis. Two different cleavable linkers were used (Fig. 2) by which individual PNA sequences could be removed from the membrane after completed synthesis. Prior to PNA synthesis, the amino-functionalised membrane was derivatised with the tetra-peptide spacer Lys[Boc]-Glu[OtBu]-εAhx-εAhx using Fmoc-chemistry. The spacer length had been selected to reduce steric interference between the PNA chains and the polymer on the membrane. For a release of the eventual oligomer, the lysyl–glutamic acid bond within this linker molecule could be cut with trypsin, the resulting PNA compounds bearing a lysine residue at their C-terminus. In few cases, the acid labile Rink-amide handle (17) was added to the tetra-peptide by an additional synthesis step to provide an alternative target for chemical cleavage. With either molecule, the loading of linkers on the membranes was about identical to the initial 70 nmol/cm^2 of amino functions present according to the manufacturer. This was determined by a bromophenol blue treatment of the membranes (16). The colour intensities indicated the number of reactive amino groups present at each position. The proceeding PNA synthesis was also continuously monitored by this method after each synthesis cycle. Moreover, a few spots were reacted with an activated solution of rhodamine during every synthesis

cycle; in addition to data on the efficiency of synthesis, this reaction generated orientation marks on the membranes.

Separate control experiments on the quality of the activated PNA mixtures had been done. An activation with azabenzotriazolyl-*N*,*N*,*N*′,*N*′-tetra-methyluronium hexafluoro-phosphate (HATU), as suggested by the manufacturer (PerSeptive manual on the Expedite synthesiser) led to a nearly complete loss of reactivity after >15 min of activation. As the distribution of 1000 spots takes ~30 min, a different activation scheme was applied (20). PNA monomers were activated with DIC and HOAt in dry NMP and applied as 0.16 M solution. After 5–10 min of pre-activation time, 0.4 µl drops were distributed to the membrane spots. Reaction took place for at least 30 min. The somewhat larger volume of 0.4 µl as compared to the 0.3 µl used during linker attachment was applied in order to avoid uncomplete coupling at the border of the spots. After completion of the synthesis process, the PNA oligomers were side-chain deprotected by submerging the dry membranes in a mixture of 90% TFA, 5% water and 5% triethylsilane. Arrays of some 1000 PNA sequences of a length of up to 16 nucleotides (nt) could be synthesised with this refined protocol.

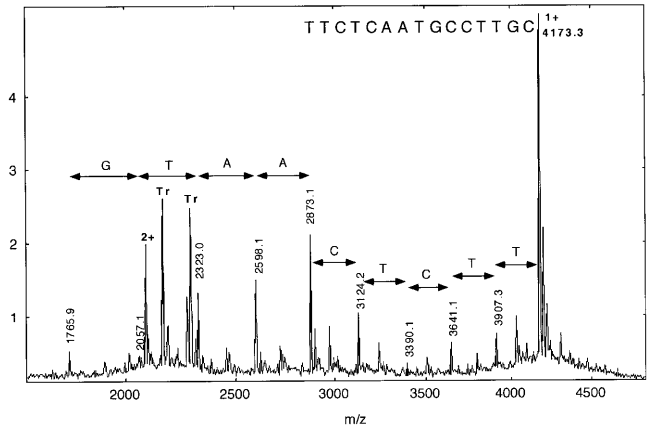


Figure 3. MALDI mass spectrum of the PNA sequence NH_2-TTCTCAATGCC-TTGC-H (M_r 4172.76). The singly (1+) and doubly (2+) charged ion signals from this PNA molecule are evident and confirm that the desired product was synthesised. Additionally, some termination products are labelled which allow a read-out of the N-terminal part of the PNA sequence for quality control. Trypsin autolysis products are also indicated (Tr).

PNA oligomer quality

During the SPOT synthesis, the colour intensity resulting from the bromophenol blue assay weakened slowly with the increasing number of synthesis cycles. Compared to original starting material, the colour intensity was >50% after completion of the synthesis of 16mer PNA molecules, for example. This figure translates into an average coupling yield of 95–96% and is attributed to steric effects (15). The colour intensity of the spots was uniform across the membranes with no indication of local differences in reactivity. Slightly better results still were obtained, when the monomer solutions were diluted to a concentration of 0.1 M and spotted twice during each cycle. In this way, stepwise coupling rates of >97% were obtained. Similar synthesis efficiency values could be concluded from the rhodamine reactions. The yield of an *in situ* PNA synthesis on membranes is therefore comparable to standard solid-phase procedures on polystyrene supports.

For mass spectrometry analyses some spots were cut out. Oligomers were released enzymatically by a trypsin digestion or, as a control in a few cases, chemically with TFA. Since the TFA labile handle is being cut during side chain deprotection, the merely trypsin cleavable linker was used in all other experiments reported here. Characterisation of the released PNA products by MALDI-TOF spectrometry confirmed a high purity as suggested by the bromophenol blue assay. A typical example of a MALDI mass spectrum of a released PNA oligomer is shown in Figure 3. The major signal in the mass spectrum originated from the desired product (calculated M_r 4172.76) and confirms successful synthesis. A series of minor signals correspond to intermediate termination products and allowed part of the PNA sequence to be determined. This is a convenient way of quality control of the released product; no modifications or internal sequence deletions could be detected.

Hybridisation of DNA fragments to PNA arrays

For the hybridisation studies, mostly PNA grids of 384 or 576 different sequences were generated by the SPOT method. In some cases, few oligomers were removed for analysis by MALDI-TOF spectrometry before the arrays were used in hybridisation experiments. The length of the synthesised PNA oligomers varied between 5 and 16 bases. In some instances, identical sub-patterns were generated on one filter (e.g., nine fields of 64 oligomers each) and cut up for individual analyses. Evaluation of the signal intensities obtained on such twin filters by identical probes also demonstrated good uniformity of synthesis across the membranes. The accessibility of N- and C-termini was confirmed by hybridisations of short DNA oligomers.

The linkage of the PNA oligomers to the membranes was found to be very stable. No apparent decrease in signal intensities could be detected even after 30 subsequent hybridisations; all membranes produced to date are still in use. Even repeated TFA treatments for the removal of unspecific but extremely stable complexes occasionally formed by hybridisation probes and homo-G PNA sequences (see below) had no negative effect.

Duplex stability

For an initial analysis of the binding and dissociation behaviour of DNA probes to arrayed PNA oligomers, pools of different 21mer DNA oligomers were hybridised under physiological conditions (37°C, 100 mM Na^+) to an array containing 64 16mer PNA sequences that represent genes of the budding yeast genome. Under such conditions, a lot of unspecific binding occurred besides the annealing to the full match sequences (Fig. 4a). Better discrimination was obtained by subsequent washes at gradually increased temperatures (Fig. 4b–d). From such experiments, the dissociation temperatures (T_d) of the full-match PNA–DNA duplexes were determined to range between 70 and 80°C, the actual value seemingly dependent on the base composition. Identical hybridisations were done to an array bearing the 64 corresponding DNA oligomers. No background signal was obtained at 37°C and the experimentally determined range of T_d of the full-match DNA–DNA duplexes was 50–55°C (data not shown). Comparable to results on PNA–DNA interaction in solution, the duplex stabilities on solid support of a complex

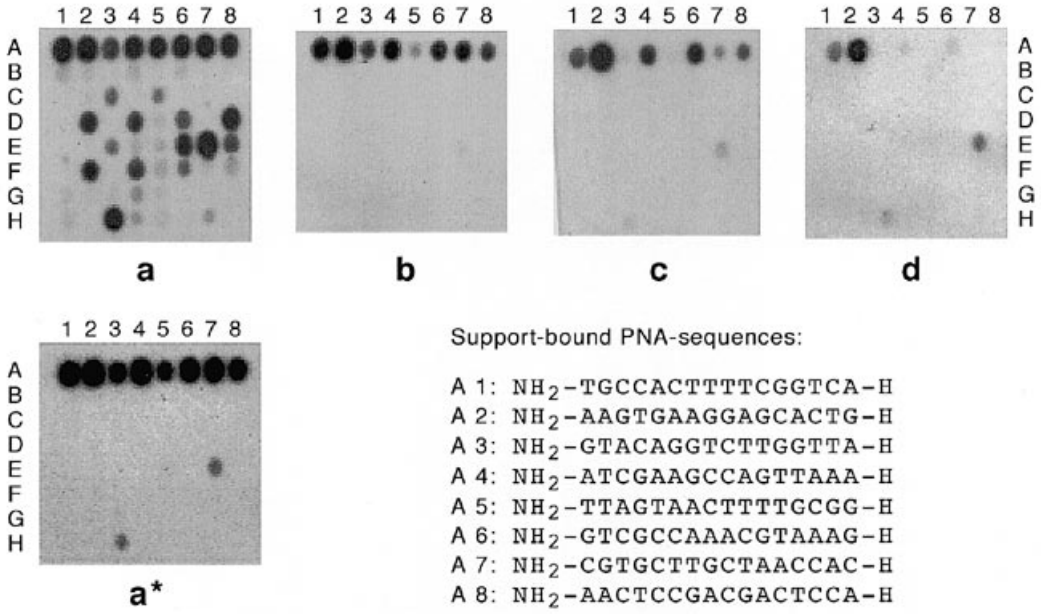


Figure 4. DNA hybridisation to PNA arrays. A PNA grid containing a set of 64 different 16mer sequences complementary to some cell cycle regulated genes of the yeast *Saccharomyces cerevisiae* was hybridised with a mixture of eight radioactively labelled 21mer DNA probes. The PNA oligomers positioned in the first row were entirely complementary to the probes, while the other spots contained completely different sequences. Hybridisation was carried out in 60 mM NaCl, 6 mM sodium citrate, pH 7.5, and 0.7% sodium *N*-lauroylsarcosine (adding up to a Na^+ concentration of 90 mM) at 37°C (**a**). Subsequent washes in gradually heated buffer [e.g., 67°C (**b**), 74°C (**c**) and 80°C (**d**)] increased the probe discrimination. In (**a***), hybridisation was carried out at 55°C instead of 37°C. No further washing at higher temperatures was necessary under these more stringent conditions.

formed by a DNA probe and a surface-attached PNA oligomer of mixed base composition can be described as being in general 1–1.5°C higher per base pair than that of the corresponding DNA–DNA hybrid.

Experiments on the kinetics showed that the specific hybridisation process of DNA probes to arrays of 16 nt long PNA sequences was at equilibrium within some 30 min. Extending the annealing period substantially, for example to overnight, only led to higher background signal and more unspecific binding of mismatched sequences. Best hybridisation results were usually obtained when hybridisation was for ~1 h at a temperature not much below the actual T_d followed by a wash at the same temperature (Fig. 4a*). In the experiments described here, a temperature of 10°C to, at most, 20°C below T_d was used.

Our results indicate that the stability of PNA–DNA duplexes longer than ~15 bp can be compared on the basis of their base composition, since they correlate with the T_d determined by empirical rules for the corresponding DNA–DNA hybrids [Fig. 5; GC-content (22); nearest neighbour analysis (23)], although the absolute T_d values are very different. Similar to the predictions of DNA stabilities, there is an increasing error for sequences shorter than 15 bp or certain sequence features, such as homonucleotide sequences. Even for 16mers of mixed base composition, however, relatively large discrepancies were found; the oligomers B8 and B9 of Figure 5, for example, differ considerably in their stability, although no such effect was predicted. Currently, further experiments are in progress on complete libraries of short PNA oligomers (e.g., all 1024 pentamer sequences) for an unambiguous and fully quantitative determination of PNA–DNA duplex stabilities.

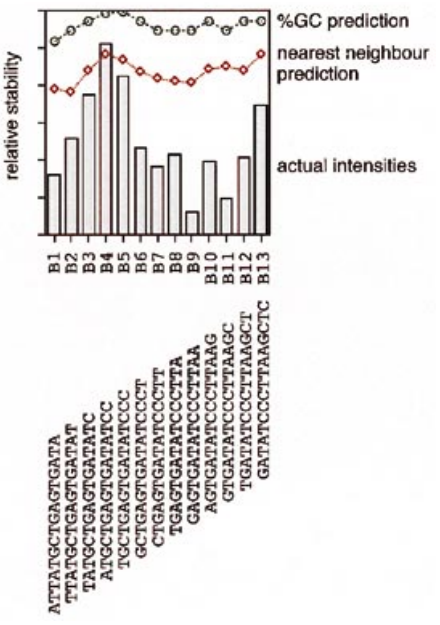
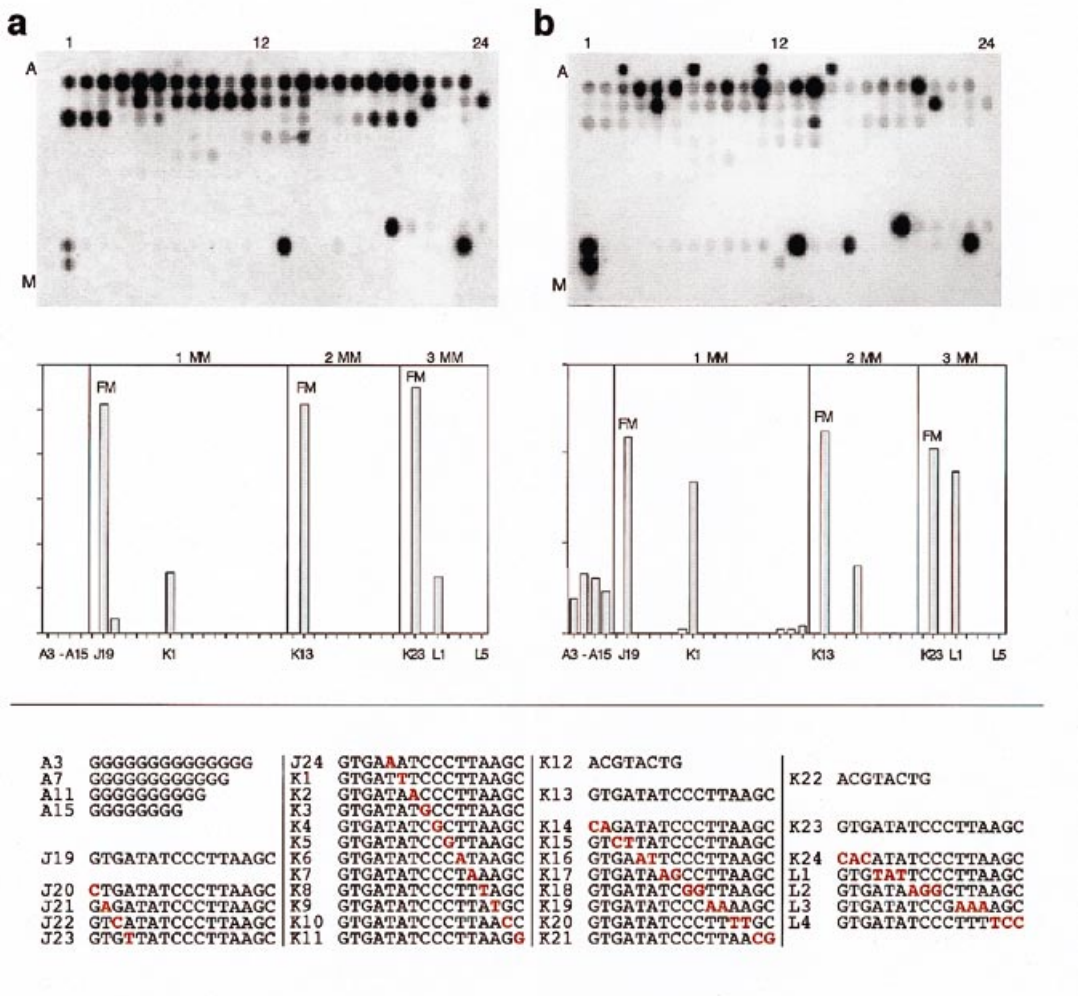


Figure 5. Prediction of the variation in thermal stability of PNA–DNA interactions. Actual binding intensities obtained with the 29mer DNA probe CGAGCTTAAGGGATATCACTCAGCATAAT on arrayed 16mer PNA complements were compared to the variation of T_d values predicted for the corresponding DNA–DNA duplexes. Hybridisation of the DNA probe to the PNA oligomers (positions B1–B13 on a grid as shown in Fig. 6) was at 55°C in 60 mM NaCl, 6 mM sodium citrate, pH 7.5, and 0.7% sodium *N*-lauroylsarcosine (totalling 90 mM Na^+).



A3	GGGGGGGGGGGGGG	J24	GTGAAATCCCTTAAGC	K12	ACGTACTG		
A7	GGGGGGGGGGG	K1	GTGATTTCCCTTAAGC			K22	ACGTACTG
A11	GGGGGGGGG	K2	GTGATAACCCTTAAGC	K13	GTGATATCCCTTAAGC		
A15	GGGGGGGG	K3	GTGATATGCCTTAAGC				
		K4	GTGATATCGCTTAAGC	K14	CAGATATCCCTTAAGC	K23	GTGATATCCCTTAAGC
		K5	GTGATATCCGTTAAGC	K15	GTCTTATCCCTTAAGC		
J19	GTGATATCCCTTAAGC	K6	GTGATATCCCATAAGC	K16	GTGAATTCCCTTAAGC	K24	CACATATCCCTTAAGC
		K7	GTGATATCCCTAAAGC	K17	GTGATAAGCCTTAAGC	L1	GTGTATTCCCTTAAGC
J20	CTGATATCCCTTAAGC	K8	GTGATATCCCTTTAGC	K18	GTGATATCGGTTAAGC	L2	GTGATAAGGCTTAAGC
J21	GAGATATCCCTTAAGC	K9	GTGATATCCCTTATGC	K19	GTGATATCCCAAAAGC	L3	GTGATATCCGAAAAGC
J22	GTCATATCCCTTAAGC	K10	GTGATATCCCTTAACC	K20	GTGATATCCCTTTTGC	L4	GTGATATCCCTTTTCC
J23	GTGTTATCCCTTAAGC	K11	GTGATATCCCTTAAGG	K21	GTGATATCCCTTAACG		

Figure 6. Hybridisation of the DNA probe CGAGCTTAAGGGATATCACTCAGCATAAT to an array of 345 PNA oligomers. Positions B1–B14 represent all full-match 16mers that cover the entire 29mer DNA probe, the sequences at B15–C5 represent all full-match 15mers, and so on and so forth. At I18–J17, finally, all respective hexamer sequences were located. Apart from the full complements, derivatives of the oligomer B11 with 1–3 consecutive mismatched bases at various positions along the sequence were synthesised. As an internal control of reproducibility, the oligomer sequence at B11 had also been produced at several other positions on the grid (J19, K13 and K23). Spots K12, K22 and a few others contained unrelated PNA sequences. Hybridisation was carried out at 55°C in 60 mM NaCl, 6 mM sodium citrate, pH 7.5, and 0.7% sodium *N*-lauroylsarcosine (totalling 90 mM Na^+) (**a**) or in the same hybridisation buffer diluted 1:10 in water (**b**). In the (partial) list of PNA oligomer sequences mismatches to the 29mer DNA probe are printed in red.

Specificity

The concentration of salt and the presence of detergency significantly influenced the specificity of the hybridisation process on the solid support, especially with regard to the discrimination between full-match and mismatch duplexes. For such analyses, DNA probes of 10–30 nt in length were hybridised to grids of 16mer PNA sequences. Apart from fully complementary 16mers, the membranes also contained sets of truncated oligomers down to a length of six bases as well as corresponding oligomers with mismatch sequences at various locations along the PNA molecule (Fig. 6). Hybridisation was carried out at different concentrations of Na^+ and *N*-lauroylsarcosine. Usually, good discrimination was obtained above 90 mM Na^+ and in the presence of *N*-lauroylsarcosine (Fig. 6a). However, a few mismatched sequences already showed an unexpected duplex stability at this and higher salt concentration. The signal intensity ratio of the strongest mismatch sequences identified by us versus the full complement was about a third to a quarter (K1 and L1 versus J19) under these conditions. Lowering the concentrations of either or both buffer components, the salt or the lauroylsarcosine, caused a reduction in the binding specificity of some sequences with decreasing concentration. Still, mostly specific binding could be detected (data not shown).

At a salt concentration below ~10 mM, the signal intensities of a DNA probe on the set of fully complementary PNA oligomers showed significant differences (Fig. 6b). More mismatched duplex formation occurred, although there was relatively good discrimination still between most full-match and mismatch duplexes. For others, however, there was strong binding even to PNA oligomers that contained up to three continuous mismatches (Fig. 6b). Surprisingly, these mismatched duplexes were found to be stable even if they were located in the centre of the oligomer. Such central mismatches are supposedly the weakest ones possible due to the only short specific regions of complementary sequence remaining at either side. The PNA sequences at the grid positions K1, K16 and L1 in Figure 6b, representing a T·T single, a (AT)·(AT) double and a (TAT)·(TAT) triple mismatch, respect-

ively, were the strongest ones found, with K1 and L1 being nearly as stable as the full-matches (J19, K13 and K13) under these conditions. Applying more stringent washing conditions could not improve the discrimination either. Hybridisations with different DNA probes indicated that the PNA–DNA mismatch stability was not dependent on the location within the duplex but on the sequence as such; other, in part different, oligomers containing the sequence region around the mismatch exhibited the same effect. Up to now we have no explanation for this effect. A similar phenomenon of stronger binding of certain mismatches compared to the respective full-match sequence has been reported for few DNA–DNA duplexes (24), the reasons for this also being unclear.

Under discriminative condition, no significant signal intensities could be detected by hybridisations of DNA probes of a sequence identical in orientation to the PNA oligomers present on the membranes, such as a hybridisation of the 29mer DNA sequence 5′-TAATACGACTCACTATAGGGAATTCGAGC-3′ to the array used in the experiments documented in Figures 5 and 6. At very low temperature some binding occurred but was most likely due to antiparallel interaction between partial sequences. This demonstrates that on arrayed PNA molecules a formation of parallel oriented PNA–DNA complexes is clearly disfavoured versus the antiparallel duplex configuration.

Apart from the mismatching events, entirely unspecific adhesion of DNA probes to homopurine and especially homo-G sequences could be observed at very low salt concentrations (Fig. 6b; A3, A7, A11 and A15). These complexes could not be denatured even in boiling buffer. Only a treatment with 90% TFA in water, which causes depurination of the DNA, led to the removal of the probe. An explanation for this result might be the known effect that certain PNA–DNA complexes tend to precipitate at low pH and in the absence of counterions (PerSeptive manual on PNA). Results very similar to those obtained at low salt concentration took place in the absence of *N*-lauroylsarcosine, even at a Na^+ concentration of 100 mM for example.

Analysis of PCR products

For potential applications of PNA arrays, such as their use as a tool for the optimisation of PNA sequences for antisense strategies, expression profiling by hybridising complex mRNA probes to oligomer grids that represent gene sequences, or even the sequence determination of a DNA fragment by hybridisation to a comprehensive set of oligomer sequences, the use of long DNA fragments as probes is important. During such studies, we also tested different fluorescent labelling techniques for their suitability on the PNA membranes. Direct labelling of the probe with infrared dyes, fluorescein and the AttoPhos™ system, all gave results that were comparable to radioactive labelling of the probe or even produced lower background.

PCR fragments of various origin were hybridised to arrays of PNA oligomers that were contained in the respective sequences. The binding stability was compared to those obtained with short DNA oligonucleotide probes. In all cases examined, no apparent influence of the position of the complement within a PCR product could be found. However, weak binding to few apparently unrelated sequences could be observed again (Fig. 7). Some of these events (e.g., at grid position C6 in Fig. 7a and B5 in Fig. 7b) were probably due to homopurine stretches within the sequence; for the others no apparent reason for hybridisation could be identified. Unspecific binding, however, could be discriminated from the respective full-match signals.

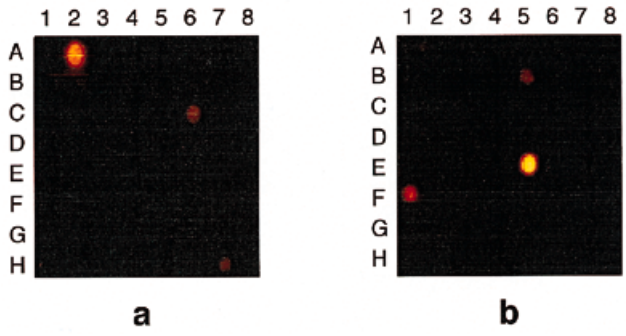


Figure 7. Fluorescence detection of a hybridisation of a 528 bp PCR product (concentration 15 ng/ml) to PNA arrays. Full-match positives are coordinates A2 (**a**) and E5 (**b**). The weakly positive oligomers at C6 and B5 contained the purine-rich stretches AAAAGCAA and GAGGGGA, respectively.

DISCUSSION

A technique for an efficient, parallel synthesis of large numbers of different PNA oligomers in nanomole amounts was established which, as an option, can be removed from the grids as intact molecules by enzymatic cleavage. Apart from the apparent utilisation of this set-up as a device for the production of very many PNA oligomers, diverse PNA arrays for hybridisation studies can be generated this way. An application as a tool in screening assays of DNA fragments would gain most, if performed at low salt concentrations. Observations by Tomac *et al.* (25) suggested that this should be possible: in solution, a decrease of the concentration of monovalent salt ions coincided with a slight increase in melting temperature of an antiparallel PNA–DNA duplex; this was explained by electrostatic effects and is in contrast to the formation of DNA–DNA duplexes.

Hybridisation to the PNA arrays occurred following the Watson–Crick base pairing rules. In agreement to experiments in solution, array-bound PNA oligomers exhibited a higher affinity to DNA probes than the corresponding DNA oligonucleotides. However, both the affinity and specificity of DNA hybridisation to the PNA oligomers depended on the hybridisation conditions more than previously expected. Successful discrimination between full complementary short PNA sequences and truncated or mismatched sequences was possible at salt concentrations down to ~10 mM salt. However, there was an increasing tendency to unspecific DNA binding. Also, the presence of *N*-lauroylsarcosine was required for discriminative hybridisation. Certain very strong binding mismatch sequences were identified even at highly stringent conditions that, subsequent to duplex formation, could not be eliminated by more stringent washing conditions, but it is as yet unclear why they exhibit such a tremendous stability; kinetic effects might be an explanation and are being investigated. Exceptionally strong and entirely unspecific complexes on G-rich PNA sequences occurred below a concentration of 10 mM Na^+. This complexation could be the consequence of precipitation.

Further experiments are in progress on comprehensive libraries of all 1024 pentamer PNA sequences and related sets of heptamer oligomers. Only from such analyses, a complete understanding of

the PNA–DNA interaction will be possible. Error-prone sequences will be identified and tested for methods to circumvent the respective problem. Also, effects such as the influence of dangling ends (26) on the duplex stability need to be studied. Nevertheless, our experiments indicate the potential of PNA oligomer arrays. For some applications, a levelling of stability would be advantageous (27). Recent investigations on modified PNA monomers containing methylene backbone extensions or other modifications (28) indicate that such modulation could be possible.

The analyses with PCR products yielded effective discrimination on immobilised PNA oligomers, with very few exceptions. Thus, PNA arrays might be a powerful tool for investigations in antisense studies for the optimisation of the chosen sequences. This seems to be of special importance because of the large degree of mismatch hybridisation detected at physiological, low stringency conditions (100 mM Na^+, 37°C), although the data shown here were produced with DNA and might not quite reflect PNA–RNA interaction. Screening for genetic mutations (29) or analysing gene expression patterns by hybridising very complex probes like total mRNA to oligomer grids (30) are alternative applications, particularly due to the large increase in hybrid stability and thus sensitivity as compared to arrays of DNA oligonucleotides. Arrays of >6000 spots could be generated with little modifications to the current set-up; these would represent the complete set of yeast genes, for example. Beside their other advantages, it seems that PNA arrays can be re-used much more often than conventional oligonucleotide arrays, since the PNA molecules are extremely stable under conditions which natural DNA cannot withstand.

ACKNOWLEDGEMENTS

The excellent technical assistance of Sandra Schwarz is gratefully acknowledged. We thank Helmut Blöcker for his help in initiating this project. The work was funded in part by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Forschung und Technologie (BMBF) and the European Commission.

REFERENCES

1 Goffeau,A. (1997) *Nature*, **385**, 202–203.
2 Timofeev,E.N., Kochetkova,S.V., Mirzabekov,A.D. and Florentiev,V.L. (1996) *Nucleic Acids Res.*, **24**, 3142–3148.
3 Lamture,J.B., Beatie,K.L., Burke,B.E., Eggers,M.D., Ehrlich,D.J., Fowle,R., Hollis,M.A., Koscicki,B.B., Reich,R.K., Smith,S.R., *et al.* (1994) *Nucleic Acids Res.*, **22**, 2121–2125.
4 Schena,M., Shalon,D., Davis,R.W. and Brown,P.O. (1995) *Science*, **270**, 467–470.
5 Maskos,U. and Southern,E. (1992) *Nucleic Acids Res.*, **20**, 1679–1684.
6 Matson,R.S., Rampal,J., Pentoney,S.L., Anderson,P.D. and Coassin,P. (1995) *Anal. Biochem.*, **224**, 110–116.
7 Chee,M., Yang,R., Hubbell,E., Berno,A., Huang,X.C., Stern,D., Winkler,J., Lockhart,D.J., Moris,M.S. and Fodor,S.P.A. (1996) *Science*, **274**, 610–614.
8 Weiler,J. and Hoheisel,J.D. (1996) *Anal. Biochem.*, **242**, 218–227.
9 Hoheisel,J.D. (1994) *Trends Genet.*, **10**, 79–83.
10 Southern,E.M. (1995) *Electrophoresis*, **16**, 1539–1542.
11 Southern,E.M., Case-Green,S.C., Elder,J.K., Johnson,M., Mir,K.U., Wang,L. and Williams,J.C. (1994) *Nucleic Acids Res.*, **22**, 1368–1373.
12 Nielsen,P.E., Egholm,M., Berg,R.H. and Buchardt,O. (1991) *Science*, **254**, 1497–1500.
13 Egholm,M., Buchardt,O., Christensen,L., Behrens,C., Freier,S.M., Driver,D.A., Berg,R.H., Kim,S. K., Norden,B. and Nielsen,P.E. (1993) *Nature*, **365**, 556–568.
14 Nielsen,P.E. (1995) *Annu. Rev. Biophys. Biomol. Struct.*, **24**, 167–183.
15 Frank,R. (1992) *Tetrahedron*, **48**, 9217–9232.
16 Krchnak,V., Vagner,J. and Lebl,M. (1988) *Int. J. Pept. Protein Res.*, **32**, 415–416.
17 Bernatowicz,M.S., Daniels,S.B. and Köster,H. (1989) *Tetrahedron Lett.*, **30**, 4645.
18 Jensen,O.N., Shevchenko,A. and Mann,M. (1997) In Creighton,T.E. (ed.), *Protein Structure - A Practical Approach, 2nd Edition*. Oxford University Press, Oxford, UK pp 29–57.
19 Maier,E., Crolius,H. and Lehrach,H. (1994) *Nucleic Acids Res.*, **22**, 3423–3424.
20 Gausepohl,H., Weiler,J., Schwarz,S., Fitzpattrick,R. and Hoheisel,J.D. (1997) In Ramage,R. and Epton,R. (eds), *Proceedings of the 24th Symposium of the European Peptide Society*. ESCOM Press, in press.
21 Pirrung,M.C. and Bradley,J.-C. (1995) *J. Org. Chem.*, **60**, 6270–6276.
22 Itakura,K., Rossi,J.J. and Wallace,R.B. (1984) *Annu. Rev. Biochem.*, **53**, 323–356.
23 Breslauer,K.J., Frank,R., Blöcker,H. and Marky,L.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3746–3750.
24 Maskos,U. (1991) A novel method of nucleic acid sequence analysis. Thesis, New College, University of Oxford, UK.
25 Tomac,S., Sarkar,M., Ratilainen,T, Wittung,P., Nielsen,P., Norden,B. and Gräslund,A. (1996) *J. Am. Chem. Soc.*, **118**, 5544–5552.
26 Williams,J.C., Case-Green,S.C., Mir,K.U. and Southern,E. (1994) *Nucleic Acids Res.*, **22**, 1365–1367.
27 Hoheisel,J.D. (1996) *Nucleic Acids Res.*, **24**, 430–432.
28 Hyrup,B., Egholm,M., Nielsen,P.E., Wittung,P., Norden,B. and Buchardt,O. (1994) *J. Am. Chem. Soc.*, **116**, 7964–7970.
29 Carlsson,C., Jonsson,M., Norden,B., Dulay,M.T., Zare,N., Noolandi,J., Nielsen,P., Lap-Chee,T. and Zielenski,J. (1996) *Nature*, **380**, 207.
30 Lockhart,D.L., Dong,H., Byrne,M.C., Follettie,M.T., Gallo,M.V., Chee,M.S., Mittmann,M., Wang,C., Kobayashi,M., Horton,H. and Brown,E.L. (1996) *Nature Biotechnol.*, **14**, 1675–1680.