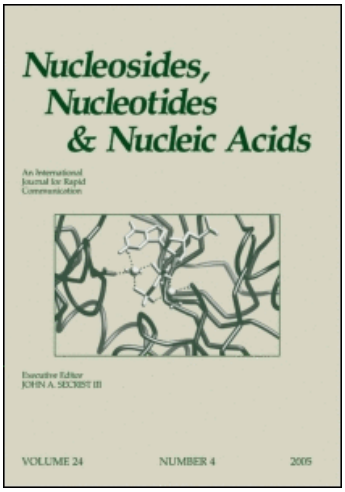

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Pna Array Technology in Molecular Diagnostics

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PNA ARRAY TECHNOLOGY IN MOLECULAR DIAGNOSTICS

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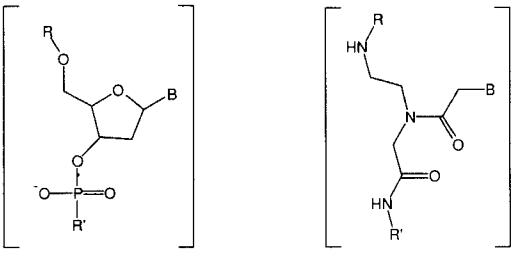
ABSTRACT: A comparative study using immobilised DNA and PNA oligomers demonstrates the suitability of PNA molecules as sequence specific capture probes in the detection of single point mutations in a DNA analyte and in the analysis of complex analyte mixtures.

INTRODUCTION

Arrays of probes immobilised on a solid support have attracted considerable interest as a convenient format for the rapid analysis of nucleic acids carrying multiple mutations[1]. Several test strips for the analysis of known mutations, e.g. in the *rpo* gene of Mycobacterium tuberculosis[2], are commercially available. Currently, microfabricated devices containing arrays of DNA probes are being developed[3,4], e.g. for the analysis of mutations in the HIV-1 drug resistance gene[5], mutations in the BRCA1 gene[6], and the analysis of gene expression[7,8]. The basic requirement for a functional array system is the ability of all different probes to hybridise to their target sequences with high specificity at a single temperature. Using DNA probes, this can roughly be achieved, either by varying the position and the length of the probes or by adding high molar concentrations of the quaternary salt TMAC to the hybridisation buffer[9].

Although DNA probes have been used successfully in many cases, their use is complicated by the fact that the buffer conditions required for probe / target hybridisation also allow the target to participate in unwanted hybridisation events, e.g. formation of intramolecular structures in the target or rehybridisation of target and complementary

strand when dsDNA is used as analyte. These unwanted side reactions can seriously limit the access of the probe to its target sequence, resulting in loss of signal intensity.



DNA PNA

A solution to this problem is to use probes with physical properties sufficiently different from those of nucleic acids to allow hybridisation of only the probe and the correct target region. This requirement is fulfilled by the recently developed DNA mimic, **P**eptide **N**ucleic **A**cid (**PNA**). By virtue of its neutral backbone, PNA can hybridise to nucleic acids in the absence of the counterions needed to stabilise pure nucleic acid duplexes[10,11]. In addition to this important property, PNA exhibits superior hybridisation characteristics (affinity and specificity) compared to DNA[11,12], thus fulfilling the second requirement for an optimal probe. Recently, synthesis and hybridisation properties of membrane bound PNA have been described[13].

RESULTS AND DISCUSSION

A set of three PNAs (PNA 1-3, 15 bases each) was used to establish a PNA reverse dot-blot format for the detection of single point mutations in oligonucleotide (ODN) analytes and for the semiquantitative analysis of mixtures of oligonucleotides (ODN 1-3). As depicted in table 1, the PNAs are either fully complementary to the corresponding analyte or contain single or double mismatches. As indicated by the corresponding T_m values, the duplexes are strongly influenced by the number of mismatches included in the duplex.

Mismatch Discrimination Property of PNA

From DNA probes it is known, that the hybridisation rate constant *k* increases with the hybridisation temperature T_i and reaches a broad maximum about 20-25°C below the T_m of the corresponding duplex[14]. At this temperature, however, the ability of the probe to discriminate between matched and single base mismatched target sequences is usually found to be relatively poor. Increasing T_i improves the discrimination ratio, but depresses

TABLE 1: Possible PNA / DNA hybrids formed between immobilised PNAs and the oligonucleotide (ODN) analytes. Mismatches are indicated in bold italic. In DNA / ODN hybridisation experiments, $(dT)_{15}$ tailed probes were used. Melting temperatures were measured using 2 nmoles of each strand in 1ml buffer containing 100mM NaCl, 10mM Na_2HPO_4 pH 7, 0.1mM EDTA.

PNA/DNA No.	Sequence	Tm (°C)
PNA 1	H-ado$_6$ -TGT ACG TCA CAA CTA-Gly-NH_2	69
ODN 1	3'-ACA TGC AGT GTT GAT-5'-Dig	
PNA 2	H-ado$_6$ -TGT ACG T***G***A CAA CTA-Gly-NH_2	55
ODN 1	3'-ACA TGC A***G***T GTT GAT-5'-Dig	
PNA 3	H-ado$_6$ -TGT AC***A*** TCA CAA CTA-Gly-NH_2	54
ODN 1	3'-ACA TG***C*** AGT GTT GAT-5'-Dig	
PNA 1	H-ado$_6$ -TGT ACG T***C***A CAA CTA-Gly-NH_2	50
ODN 2	3'-ACA TGC A***C***T GTT GAT-5'-Dig	
PNA 2	H-ado$_6$ -TGT ACG TGA CAA CTA-Gly-NH_2	70
ODN 2	3'-ACA TGC ACT GTT GAT-5'-Dig	
PNA 3	H-ado$_6$ -TGT AC***A*** T***C***A CAA CTA-Gly-NH_2	32
ODN 2	3'-ACA TG***C*** A***C***T GTT GAT-5'-Dig	
PNA 1	H-ado$_6$ -TGT AC***G*** TCA CAA CTA-Gly-NH_2	60
ODN 3	3'-ACA TG***T*** AGT GTT GAT-5'-Dig	
PNA 2	H-ado$_6$ -TGT AC***G*** T***G***A CAA CTA-Gly-NH_2	44
ODN 3	3'-ACA TG***T*** A***G***T GTT GAT-5'-Dig	
PNA 3	H-ado$_6$ -TGT ACA TCA CAA CTA-Gly-NH_2	66
ODN 3	3'-ACA TGT AGT GTT GAT-5'-Dig	

k severely, and at temperatures near or at T_m the value of *k* becomes impractical low. As observed with DNA, the hybridisation rate constant *k* for PNA also shows a maximum around 25°C below the T_m. As a result of its increased specificity, however, the ability of PNA to effectively discriminate between matched and mismatched target sequences at this temperature is markedly better than that of DNA. As an example, the temperature influence on the rate constant *k* for three different 15mer PNA probes differing in sequence at a single position is shown in figure 1. Some of the reverse dot-blots used to establish the graphs are also shown.

Specificity of PNA and DNA Probe Hybridisation

For PNA probe hybridisation, 10mM sodium phosphate buffer containing 0.1% SDS was found to be optimal. The ionic strength of the buffer was sufficiently low to

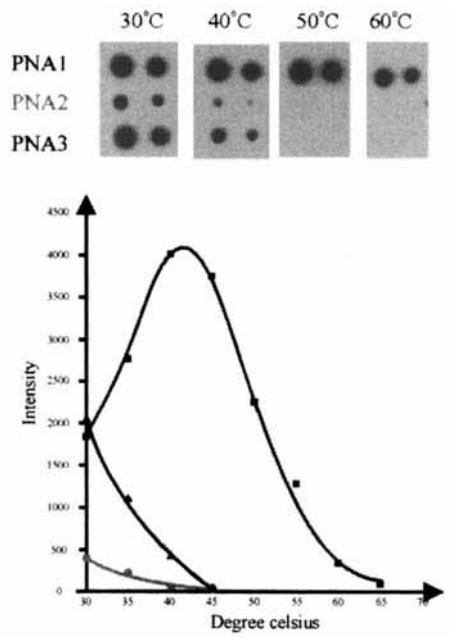


FIGURE 1: Mismatch discrimination of PNA.
1 nM solutions of ODN 1 in 10mM sodium phosphate buffer containing 0.1% SDS were hybridised at different temperatures to a PNA array consisting of a set of three different probes, each immobilised in different amounts per spot (100 pmol or 40 pmol). Spot intensities were plotted against hybridisation temperature (for the duplexes see table 1).

destabilise a 118bp PCR amplicon, thus resulting in a detectable hybridisation signal without prior denaturation (data not shown). When using DNA probes, the hybridisation temperature was lowered to 37°C and 6x SSC buffer was used for hybridisation. DNA probe hybridisation requires buffers with high salt concentrations, while PNA probes showed only little salt dependency (data not shown). For all combinations of probe and analyte, PNA probes showed better mismatch discrimination properties than DNA, even under high salt hybridisation conditions which are suboptimal for PNA. As shown in table 2, the G/T mismatch between probe 1 and ODN 3 (which is the least destabilising mismatch tested so far) could be discriminated by a factor of 4:1 when using PNA, but only by a factor of 3:2 when a DNA probe was used.

Analysis of Analyte Mixtures Using PNA Triple Arrays

Three different PNAs (table 1) were immobilised on membrane strips and hybridised for 30 minutes at 45°C at low salt buffer conditions to seven different mixtures of the

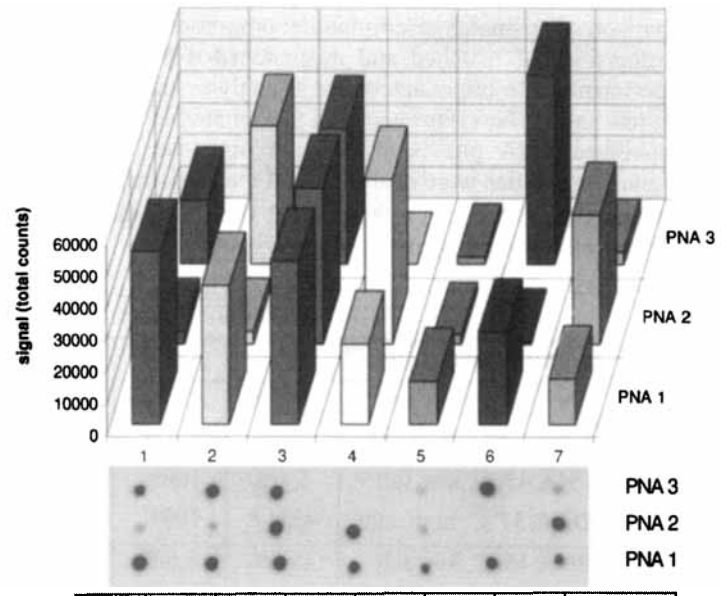
TABLE 2: Comparison of mismatch discrimination properties of PNA and DNA. Relative signal intensities of matched and mismatched duplexes were obtained from hybridisation experiments with oligonucleotides as analytes and either immobilised PNA probes at 45°C in low salt buffer (10mM sodium phosphate pH 7, 0.1% SDS, and 0.1mM EDTA) or immobilised DNA probes at 37°C in high salt buffer (6x SSC buffer). Luminescence signals intensities were analysed with the LumiImager™ and compared to the full match intensity (≡ 100%). (*) indicates that the signal intensity of the spot was less than 3x SD of the background signal.

		immobilised probe		
analyte	type of probe, buffer	1	2	3
ODN 1	PNA 45°C, low salt	100%	1,2%	1,5%
	DNA 37°C, high salt	100%	1,2%	6,4%
ODN 2	PNA 45°C, low salt	1,1%	100%	2,9%
	DNA 37°C, high salt	<2% *	100%	<2% *
ODN 3	PNA 45°C, low salt	26,0%	1,5%	100%
	DNA 37°C, high salt	66,5%	<2% *	100%

complementary oligonucleotides present in the amounts indicated in figure 2. The hybrids were detected as described and the signal intensities were extracted from the luminescence image. Very similar signals were obtained when equal amounts of all three ODNs were present in the hybridisation reaction (exp. 3). Varying the amount of any of the ODN changes the obtained signal from the corresponding probe (exp. 1, 2, 5 and 7). Omitting either ODN 3 (exp. 4) or ODN 2 (exp. 6) from the hybridisation mixture decreases the signal from the corresponding probe to below the detection limit.

Discussion

Developing rapid screening techniques for the detection of multiple sequence variations is a major focus of molecular diagnosis. Although DNA probes have been used successfully in many instances, a general solution to the problem of target sequence inaccessibility due to competing hybridisation events remains unsolved. Asymmetric PCR[15], single strand separation[1(b)] or in vitro transcription to RNA[16] have been used as means of circumventing the problem of reannealing of complementary strand and target strand. Although effective in some cases, this approach does not solve the problem of target sequence inaccessibility due to intramolecular structures.



100	1000	1000	0	10	1000	10	ODN 3 (pM)
10	10	1000	1000	10	0	1000	ODN 2 (pM)
1000	1000	1000	100	100	100	100	ODN 1 (pM)

FIGURE 2: Analysis of analyte mixtures using PNA triple arrays
20 pmoles of each of the three different PNAs were immobilised on a membrane strip and hybridised at 45°C in low salt buffer (10mM sodium phosphate pH 7, 0.1% SDS, and 0.1mM EDTA) to one of the mixtures containing the analytes in concentrations as depicted.

The data presented here demonstrate that PNA is well suited as a probe in an array format. In particular, the data demonstrate that immobilised PNA probes can effectively discriminate between single base mismatches in the target sequence at temperatures near the optimum for hybridisation kinetics. The data further demonstrate that immobilised PNA probes can hybridise to nucleic acids under ionic conditions that destabilise nucleic acid structures. Thus, the use of PNA probes offers a solution to the long standing problem of target sequence inaccessibility due to competing hybridisation events. As an extension of this feature we have found that small dsDNA amplicons can be captured on PNA arrays without prior denaturation by physical or chemical means. The data finally demonstrate that PNA arrays can be used to analyse and roughly quantify the amount of target molecules over a considerable concentration range.

MATERIAL AND METHODS

Reagents and chemicals

PNAs were synthesised using Boc/Z chemistry as described previously[17]. Oligonucleotides were obtained from Interactiva (Ulm, Germany), nylon membranes were obtained from PALL (Germany). Enzymes, buffers and reagents for luminescence detection were obtained from Boehringer Mannheim (Germany).

General Protocol for Reverse Dot Blot Using Activated Nylon Membranes

PNA or DNA probes in appropriate concentrations were covalently immobilised by adding 200nl - 400nl of probe with a pipette (Gilson P2) to an activated nylon membrane (Immunodyne ABC, PALL). After inactivation of the membrane with sodium hydroxide solution, hybridisation to a single oligonucleotide (ODN) containing solution, ODN mixture or PCR product was performed at suitable temperatures in a hybridisation oven (Hybaid Minioven 10). All analytes were Digoxigenin labelled and hybridisation detection was done using reagents and buffers from Boehringer Mannheim (BM Dig Wash and Block Buffer Set, anti-Dig AP-fab-fragment, CDP-Star™). The luminescence signal was detected either with a luminescence imaging system based on a CCD camera (Boehringer Mannheim, LumiImager™) or via autoradiography.

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