*Nucleic Acids Research, 2003, Vol. 31, No. 19* ***e119***
*DOI: 10.1093/nar/gng120*

# PNA microarrays for hybridisation of unlabelled DNA samples

**Ole Brandt*, Julia Feldner[1], Achim Stephan, Markus Schröder[1], Martina Schnölzer[2], Heinrich F. Arlinghaus[1], Jörg D. Hoheisel and Anette Jacob**

Functional Genome Analysis and [2]Protein Analysis Facility, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany and [1]Physikalisches Institut, Universität Münster, Wilhelm-Klemm-Strasse 10, 48149 Münster, Germany

Received July 9, 2003; Revised and Accepted August 16, 2003

## ABSTRACT

**Several strategies have been developed for the production of peptide nucleic acid (PNA) microarrays by parallel probe synthesis and selective coupling of full-length molecules. Such microarrays were used for direct detection of the hybridisation of unlabelled DNA by time-of-flight secondary ion mass spectrometry. PNAs were synthesised by an automated process on filter-bottom microtitre plates. The resulting molecules were released from the solid support and attached without any purification to microarray surfaces via the terminal amino group itself or via modifications, which had been chemically introduced during synthesis. Thus, only full-length PNA oligomers were attached whereas truncated molecules, produced during synthesis because of incomplete condensation reactions, did not bind. Different surface chemistries and fitting modifications of the PNA terminus were tested. For an examination of coupling selectivity, bound PNAs were cleaved off microarray surfaces and analysed by MALDI-TOF mass spectrometry. Additionally, hybridisation experiments were performed to compare the attachment chemistries, with fully acetylated PNAs spotted as controls. Upon hybridisation of unlabelled DNA to such microarrays, binding events could be detected by visualisation of phosphates, which are an integral part of nucleic acids but missing entirely in PNA probes. Overall best results in terms of selectivity and sensitivity were obtained with thiol-modified PNAs on maleimide surfaces.**

## INTRODUCTION

DNA microarray technology has come a long way, nowadays being applied in biological and biomedical research on a routine basis. Various biological aspects can be studied with this technology, of which transcriptional profiling (1) and detection of single nucleotide polymorphisms (SNPs) (2,3) are currently the most widely performed analyses. While data interpretation has already become the bottleneck of the former, the latter process is still reeling from the lack of sufficient raw data. Information on many SNPs in a large number of samples is required, for example, before associations can be detected between polymorphisms and phenotypical variations found in epidemiological studies. For other applications, such as the investigation of epigenetic variations (4), it would be advantageous to interrogate very many to possibly all methylation sites in a single experiment. To date, DNA oligonucleotide microarrays have not been able to deal with samples of such complexity, although on-chip primer extension reactions (5,6) and schemes for the preparation of appropriate hybridisation samples (see, for example, 7) have led to significant improvements. The application of peptide nucleic acids (PNAs) as arrayed probe molecules (8) could offer an alternative with superior performance. PNA oligomers are synthetic DNA mimics with an amide backbone (9,10) that exhibit several advantageous features. They are stable under acidic conditions and resistant to nucleases as well as proteases (11). Their neutral backbone increases the binding strength to complementary DNA compared to the stability of the respective DNA duplex (12,13). Thus, PNA oligomers can be shorter than oligonucleotides when used as hybridisation probes. In turn, mismatches have a more destabilising effect, thus improving discrimination. Due to their uncharged nature, PNA also permits the hybridisation of DNA samples under low or no salt conditions, since no interstrand repulsion, as occurs between two negatively charged DNA strands, needs to be counteracted. As a consequence, the target DNA has less secondary structure and is more accessible to the probe molecules. Most important, with respect to an alternative mode of detection, however, are the differences between the backbones of DNA and PNA. PNA:DNA or PNA:RNA duplexes can be visualised by time-of-flight secondary ion mass spectrometry (TOF-SIMS), detecting the phosphates that are an integral part of the nucleic acids but completely missing in PNA (14,15). The combination of PNA microarrays with TOF-SIMS detection has the potential to be a highly sensitive method for direct detection of the binding to microarrays of unlabelled RNA or DNA. However, for a robust analysis, relatively large numbers of high quality oligomers are required. In addition, they should

*To whom correspondence should be addressed. Tel: +49 6221 424679; Fax: +49 6221 424687; Email: o.brandt@dkfz.de

be arranged in a microarray format in order to decrease the reaction volume and simplify the detection process.

Generally, two approaches can be taken for the production of PNA arrays. PNA can be synthesised *in situ* in a parallel manner on porous support media (16,17) by using the Spot method (18). This procedure permits the simultaneous synthesis of many different molecules and has the advantage of a very low consumption of reagents per oligomer. However, the PNA quality is limited by the coupling efficiency of the activated monomers. The longer the PNA oligomers get, the more incompletely elongated sequences are present at each spot, thereby compromising performance. Furthermore, neither the number of spots per array nor the number of array copies that result from this procedure are sufficient for high throughput analyses. In addition, the porous support lacks mechanical stability and is therefore less suited for handling and most detection proceedings. Alternatively to *in situ* synthesis, pre-fabricated PNA molecules can be spotted onto an appropriate support medium. If HPLC-purified molecules were used, PNA arrays could be of high quality with respect to the oligomers and appropriate in dimension and format. Also, sufficient copy numbers could be produced. In contrast to oligonucleotide synthesis, however, PNA chemistry is not that widely used and therefore rather expensive. Additionally, the scale of standard synthesis is about 2 µmol, which is much more than is needed for microarray production, thus adding extra cost. Also, standard HPLC purification of many oligomers would be labour intensive and time consuming.

By combining the advantages of the two approaches and adding a purification scheme that is intrinsic to the spotting process, we present in this paper a rapid and cost-effective strategy for the production of PNA microarrays. On the basis of earlier work (8,17), parallel synthesis of different PNAs by standard Fmoc chemistry on a resin is performed on filter-bottom microtitre plates. A half-automated process using Boc chemistry has also been reported by Nielsen and colleagues (19). The much milder reaction conditions of Fmoc chemistry, however, permit a fully automated process. The amount of PNA synthesised in each well is defined by the amount of resin and can therefore be adapted to the actual requirements. In any case, it suffices for many microarray copies. Synthesis runs overnight in an automated manner with little consumption of PNA monomers and activating reagents, which are the most expensive consumables. Once released from the resin, the oligomers are easily recovered by transfer to another microtitre dish, ready for spotting without purification. Only the full-length molecules of the synthesis products are attached to the microarray surfaces by selective binding of their terminal amino, thiol or biotin groups, respectively, while shorter derivatives do not bind covalently and are washed away. This overall process yields PNA microarrays of high quality that are affordable even for arrays with very many PNA oligomers.

## MATERIALS AND METHODS

### Chemicals

Unless stated otherwise, all chemicals and solvents were purchased from Fluka (Steinheim, Germany), Sigma-Aldrich (Munich, Germany) or SDS (Peypin, France) and used without further purification. Fmoc-protected PNA monomers, {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (Fmoc-AEEA-OH linker) and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) were obtained from PE Biosystems (Framingham, MA, USA). The rink resin LS used was from Advanced ChemTech (Louisville, KY, USA). Fmoc-protected rink linker (p-[(*R*,*S*)-α-[1-(9*H*-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid) and Fmoc-Cys(Mmt) were obtained from Novabiochem-Calbiochem (Läufelfingen, Switzerland). Unlabelled and labelled oligonucleotides were purchased from Thermo Hybaid (Ulm, Germany). Pre-sliced 2 × 2 cm and 5 × 5 mm silicon wafers, gold-coated silicon wafers (both from GeSiM, Rostock, Germany) and non-derivatised microscope slides (Menzel-Gläser, Braunschweig, Germany) were used as solid supports.

### PNA synthesis

Synthesis of the PNAs was performed automatically with an AutoSpot robot (Intavis Bioanalytical Instruments AG, Cologne, Germany) in 96-well plates that have a frit in each well. A vacuum was applied to remove the reagents from the wells during the synthesis cycles. The Fmoc-protected rink resin LS (100–200 mesh, substitution of 0.2 mmol/g) was swelled for 1 h in *N*,*N*-dimethylformamide (DMF) (2 mg resin per 100 µl). The solution was thoroughly mixed and a volume of 100 µl was distributed to each well for a standard scale synthesis. After extraction of the DMF, Fmoc protection groups were removed from the resin by successive 1 and 5 min incubations with 30 µl 20% (v/v) piperidine in DMF, with one DMF washing step in between. The resin was then washed five times with 80 µl DMF followed by the first coupling reaction. Per well, a volume of 4 µl Fmoc-protected PNA monomer, biotin, AEEA-OH spacer or Cys(Mmt) [each 0.3 M in 1-methyl-2-pyrrolidone (NMP)], respectively, was activated for 60 s with 2 µl HATU (0.6 M in DMF) and a 2 µl mix of 0.6 M *N*,*N*-diisopropylethylamine (DIEA) and 0.9 M 2,6-lutidine in DMF. Subsequently, the resin in each well was incubated with this mixture at room temperature for 20 min. Coupling was repeated after rinsing with DMF in between. The resin was then washed three times with DMF. For the capping of free, non-elongated amino groups, there was incubation with 5% acetic anhydride and 6% 2,6-lutidine in DMF for 5 min. Finally, the resin was washed another five times with 80 µl DMF. Deprotection, coupling of the next monomer and capping were repeated as described above until synthesis of the PNA molecule was completed. For synthesis of fully acetylated PNAs, five additional acetylation steps were carried out after removal of the last Fmoc protection group.

Prior to cleaving the PNA products from the resin, they were washed five times with 80 µl DMF followed by three washing steps with 80 µl 1,2-dichloroethane. After the resin was dried, 100 µl cleavage mix consisting of 80% trifluoroacetic acid (TFA) with 5% triisopropylsilane in 1,2-dichloroethane were added for 1 h at room temperature. Subsequently, the PNAs were processed in two different ways. They were either eluted from the resin with 200 µl water into another microwell plate, lyophilised and each dissolved in 100 µl water. Alternatively, the PNA was eluted from the resin with another 150 µl cleavage mix and subsequently

precipitated twice with 1 ml ice-cold diethyl ether. After the remaining ether was evaporated, each PNA was dissolved in 100 µl water and stored at 4°C. For quality control, a 1 µl aliquot was diluted in 20 µl water and analysed by MALDI-TOF mass spectrometry. PNA concentrations were calculated from OD values determined at 260 nm.

### MALDI-TOF mass spectrometry

MALDI mass spectra were recorded in positive ion reflector mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik, Bremen, Germany) equipped with a 337 nm nitrogen laser. Ion acceleration voltage was 20 kV; reflector voltage 21.5 kV; first extraction plate 15.1 kV. As the matrix, 3-hydroxypicolinic acid (HPA) (0.7 M HPA, 70 mM ammonium citrate in 50% aqueous acetonitrile) was used. For quality control measurements, 0.5 µl diluted PNA solution and 0.5 µl matrix were mixed directly on the target. The mixture was allowed to dry at ambient temperature and was then introduced into the mass spectrometer for analysis.

### Microarray surface derivatisation

*General.* Unless stated otherwise, all reactions were carried out at room temperature. All reactions were performed identically for both silicon wafers and glass slides. Storage of modified supports was at 4°C. Activation with *N*,*N*′-disuccinimidyl carbonate (DSC) or 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (EMCS) was carried out directly prior to the spotting process.

*Silanisation.* Untreated silicon wafers were washed with dimethyl sulfoxide, ethanol and water and then etched in 10% NaOH (w/w) at room temperature for 1 h, followed by sonification for 15 min. After washing the wafers thoroughly with water and ethanol, they were incubated by shaking gently in a silanisation solution of 1 ml [3-aminopropyl]triethoxy silane in 20 ml 95% ethanol for 1 h and an additional 15 min in an ultrasonic bath. Finally, the wafers were rinsed twice with ethanol, once with water, dried under a stream of nitrogen and heated to 110°C for 20 min.

*Succinimidyl ester activation.* Aminosilane-modified silicon wafers were immersed in a solution made of 150 mg DSC and 0.5 ml DIEA in 14.5 ml dried acetone on a shaker at room temperature for 2 h, washed twice with 15 ml dried acetone and twice with 15 ml dichloroethane. The dried wafers were directly taken for the spotting procedure.

*Maleimide activation.* Aminosilane wafers (2 × 2 cm) were covered with 40 µl of 20 mM EMCS in a 10% solution of diisopropylethylamine in anhydrous DMF. The reaction was carried out under a coverslip at room temperature for 3 h. The wafers were washed twice with DMF and once with acetone, air dried and immediately used for spotting.

*Streptavidin coating.* Succinimidyl ester activation of aminosilane wafers was carried out as described above. After drying, each activated wafer was incubated overnight in a humid chamber with 60 µl of an aqueous solution of 1 mg/ml streptavidin in 60 mM sodium phosphate, 0.45 M sodium chloride (3× phosphate-buffered saline). The wafers were subsequently washed with 1× BW buffer (5 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl). Wafers were stored for up to 1 week in 1× BW buffer at 4°C. Prior to spotting, they were rinsed briefly with water and dried under a stream of nitrogen.

*Rink linker coating.* Prior to the reaction, Fmoc-rink linker was pre-activated for 3 min by adding 1.3 mol equivalent diisopropylcarbodiimide and 1 mol equivalent 1-hydroxy-7-azabenzotriazole and diluted to a final concentration of 0.1 M in NMP. For coupling, aminosilane-modified silicon wafers were covered with this solution (5 × 5 mm wafer, 20 µl; 2 × 2 cm wafer, 60 µl) at room temperature for 4 h. After two washings with DMF, capping was carried out by two successive incubations of 3 and 7 min, respectively, with a solution made of 240 µl acetic anhydride and 240 µl anhydrous pyridine in 20 ml DMF. Afterwards, the wafers were washed with DMF and treated twice (3 and 7 min) with 20% piperidine in DMF (v/v) in order to remove the Fmoc protection groups, followed by a final washing step with DMF. For a rink linker surface with AEEA-OH spacer, the above synthesis steps were repeated using the Fmoc-AEEA-OH compound. Finally, the wafers were washed with ethanol, dried with nitrogen and either stored at 4°C or directly activated with EMCS (for maleimide rink linker surfaces) or DSC (for succinimidyl-AEEA rink linker surfaces) as described above.

### Spotting of PNAs

PNAs were spotted at concentrations ranging from 5 to 200 µM. Depending on the surface employed, different spotting buffers were used. For maleimide-activated wafers and gold surfaces, 1 M betaine in water was used with a pH of 7.0. The same solution was used on succinimidyl-activated surfaces, but here the pH was adjusted to 7.5 with NaOH. For streptavidin surfaces, PNA was taken up in 1× BW buffer. Spotting was performed with an SDDC-2 DNA Micro-Arrayer from Engineering Services Inc. (Toronto, Canada). SMP3 pins (TeleChem International Inc., Sunnyvale, CA, USA) were used except for the spotting on streptavidin-coated slides, for which SMP10B pins were used. Centre-to-centre spot spacing was 170 µm for the SMP3 pins and 600 µm for the SMP10B pins.

After spotting, gold wafers as well as the EMCS- and DSC-treated surfaces were incubated at room temperature overnight. Succinimidyl slides were deactivated in a solution of 50 mM succinic anhydride and 150 mM 1-methylimidazole in dichloroethane (20), each slide being shaken in 15 ml for 2 h. After blocking, the slides were washed twice with dichloroethane. Maleimide surfaces and gold wafers were deactivated in 1 mM 3-mercaptopropionic acid in ethanol for 30 min, followed by three washing steps with 15 ml of ethanol, DMF and again ethanol. All surfaces were then rinsed twice with 5 mM sodium phosphate and 0.1% SDS that had been heated to 90°C, then incubated in water of the same temperature for 10 min, followed by rinsing with 1 M NaCl in 0.1% aqueous TFA and water. After drying with nitrogen, they were ready for hybridisation.

In contrast to this, streptavidin-modified surfaces were incubated in a humid chamber for 45 min directly after spotting and subsequently blocked with 1 mM biotin in 1× BW buffer with gentle shaking for 20 min. The wafers were then washed thoroughly with 1× BW buffer (three times with

**Table 1.** Sequences of PNA oligomers and DNA oligonucleotides

| Name | Sequence |
|---|---|
| *TC-PNA* | X-Li-TTCTCCCTCTCTC-Li-$CONH_2$ |
| *Seq1-PNA* | X-Li-AGCTTACGGATCA-Li-$CONH_2$ |
| *Seq2A-PNA* | X-Li-TTGAATAGCTCGA-Li-$CONH_2$ |
| *Seq2G-PNA* | X-Li-TTGAATGGCTCGA-Li-$CONH_2$ |
| *Seq2C-PNA* | X-Li-TTGAATCGCTCGA-Li-$CONH_2$ |
| *Seq2T-PNA* | X-Li-TTGAATTGCTCGA-Li-$CONH_2$ |
| *Seq3-PNA* | X-Li-TAAGTCTTAGTCATT-Li-$CONH_2$ |
| *DNA-TC-Cy5* | 5′-Cy5-GAGAGAGGGAGAA-3′ |
| *DNA-TC-long* | 5′-TTCGATAGCAGCTAAATTCTGAGAGAGGGAGAATTCTACAATAACTGCGC-3′ |
| *DNA-Seq1-Cy5* | 5′-Cy5-CTGATCCGTAAGCT-3′ |
| *DNA-Seq1-long* | 5′-AGCGATAGCAGTTTGACCATCTGATCCGTAAGCTGCGTAAGCCGTATTCC-3′ |
| *DNA-Seq2A-Cy5* | 5′-Cy5-TCGAGCAATTCAA-3′ |
| *DNA-Seq2G-Cy3* | 5′-Cy3-TCGAGCGATTCAA-3′ |
| *DNA-Seq2C-Cy5* | 5′-Cy5-TCGAGCCATTCAA-3′ |
| *DNA-Seq2T-Cy5* | 5′-Cy5-TCGAGCTATTCAA-3′ |
| *DNA-Seq2G-long* | 5′-GCTTTACCATTGATACTCTTCGAGCGATTCAATCGGTCAGAATACTTCTAC-3′ |

The DNA sequences complementary to the respective PNA (*TC*, *Seq1*, *Seq2A*, *Seq2C*, *Seq2G* or *Seq2T*) are underlined. Li=AEEA-OH linker; X=$NH_2$, acetyl, cysteine or biotin.

shaking and once in an ultrasonic bath for 15 min) and then stored in 1× BW buffer. Prior to hybridisation experiments, a short washing step with water and hybridisation buffer was carried out.

### Cleavage from the surface

In order to check binding selectivity via MALDI-TOF analysis, maleimide-activated surfaces with rink linker modification, succinimidyl-activated wafers with an AEEA and rink linker as well as strepatvidin-coated slides (in each case 5 × 5 mm silicon slides) were covered with 15 µl of the corresponding spotting solution containing 50 µM PNA. Incubation, blocking and washing procedures were carried out as described above. For the rink linker-modified surfaces, cleavage occurred by incubation in a mixture of 95% TFA and 5% triisopropylsilane for 40 min. Streptavidin slides were heated to 70°C in 25% aqueous $NH_3$ suprapur (Merck, Darmstadt, Germany) for 20 min. The released molecules were lyophilised, dissolved in 5 µl water and concentrated with ZipTip pipette tips (Millipore Corp., Eschborn, Germany) as recommended by the manufacturer. The products were directly eluted onto a MALDI target with 1.5 µl HPA matrix and analysed. For a comparison of MALDI spectra of the crude and respective cleaved PNAs, all PNA samples were concentrated by the ZipTip procedure.

### Hybridisation

For the detection of single base mismatches, two fluorescently labelled oligonucleotides were mixed (*DNA-Seq2A-Cy5* and *DNA-Seq2G-Cy3*, *DNA-Seq2C-Cy5* and *DNA-Seq2G-Cy3* or *DNA-Seq2T-Cy5* and *DNA-Seq2G-Cy3*; see Table 1) to a final concentration of 0.2 µM each in 0.1× SSarc buffer (60 mM sodium chloride, 6 mM sodium citrate, 0.72% v/v *N*-lauroylsarcosine sodium salt solution). For the other hybridisations, three fluorescently labelled oligonucleotides (*DNA-TC-Cy5*, *DNA-Seq1-Cy5* and *DNA-Seq2G-Cy3*; see Table 1) were mixed. About 18 µl of each mixture were cast onto a 2 × 2 cm slide and covered with a coverslip. Incubation was in a hybridisation chamber (TeleChem) at 38°C for 2 h. Subsequently, the slides were washed twice with 0.1× SSarc at the same temperature, rinsed with water and dried with nitrogen.

### Microarray analysis

Fluorescence signals were detected on a ScanArray 3000 or ScanArray 5000 unit (Packard, Billerica, MA, USA) and analysed with the GenePix software package (Axon Instruments, Union City, CA, USA).

Label-free detection of bound DNA by secondary ion mass spectrometry was performed with a TOF-SIMS type IV instrument with $Ar^+$ and $Xe^+$ primary ions at energies of 10 keV. The total ion dose density was $10^{12}$ per $cm^2$ at a repetition rate of 5 kHz. The mass resolution was 6000 m/Δm.

## RESULTS

### PNA synthesis

An automated synthesis of PNA oligomers in 96-well plates was established using standard Fmoc chemistry and rink amide resin. The process was optimised with regard to reaction times, washing steps and the ratio of the amounts of resin and reagents. Performing two successive coupling steps compensated for the lack of stirring or other mixing processes within the wells. Intensive washing and longer coupling periods led to higher yields of full-length PNA oligomers (Fig. 1). Molecules of up to 22 nt in length were synthesised, with the stepwise yield being 85–98%, the variance depending on the actual sequence. As PNA synthesis is basically peptide chemistry, the introduction of amino acids or terminal modifications like biotin was easy to achieve and resulted in the desired products (see, for example, Fig. 2). The protocol detailed in Materials and Methods yielded ~100 nmol crude PNA product per well. This value could be altered to meet the actual need, however, by changing the amounts of resin and reagents correspondingly.

After synthesis, the PNAs were fully deprotected and cleaved from the resin by incubation with a mixture of TFA and triisopropylsilane in dichloroethane. If the molecules were for applications other than microarray production, they were

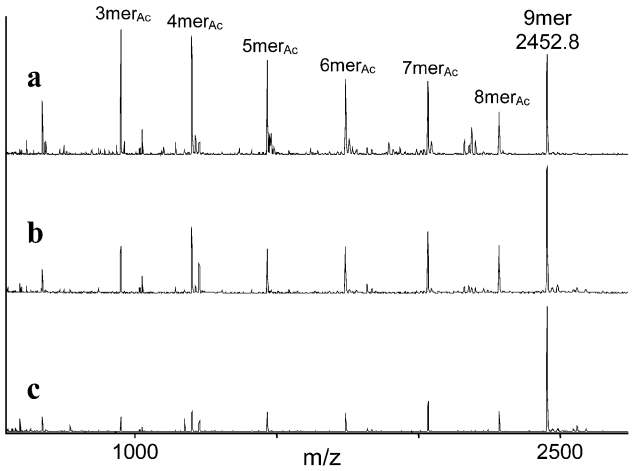


**Figure 1.** Influence of synthesis parameters on quality of the PNA $H_2N$-TCGATCAGT-$CONH_2$. (**a**) Two subsequent couplings of 10 min each were performed. There was one washing step after deprotection, one after coupling and two after capping. (**b**) Duration of the two coupling reactions was extended to 15 min and there were more washing steps: three after deprotection, one after coupling and again three after capping. (**c**) While coupling remained the same, the number of washing steps was increased to five after deprotection, three after coupling and six after capping.

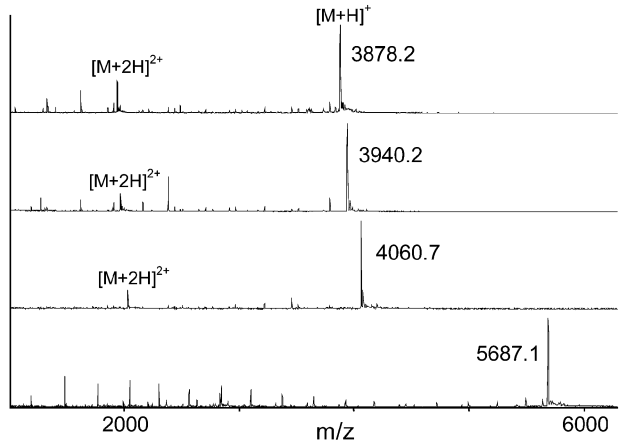


**Figure 2.** Quality control of PNA synthesis. Mass spectra of oligomer *Seq1-PNA* with different N-terminal groups are shown, obtained by MALDI-TOF mass spectrometry directly after synthesis. From top to bottom, data are presented for acetylated *Seq1-PNA* ($[M+H]^+$, $m/z$ = 3877.7), the oligomer with an N-terminal cysteine ($[M+H]^+$, $m/z$ = 3938.9) and with a biotin modification ($[M+H]^+$, $m/z$ = 4062.0), respectively. Additionally, a mass spectrum for Li-Li-CCATACAAATTTCAGGATTT ($[M+H]^+$, $m/z$ = 5686.5) (Li is an AEEA-OH linker) is presented in the bottom panel as an example of the typical quality of a 20mer PNA.

eluted from the resin with additional TFA mixture into new vials and subjected to ether precipitation for the removal of the Bhoc side chain protection groups. For the rapid and economic production of microarrays, however, ether precipitation was avoided. The PNAs were eluted from the resin after addition of some water, lyophilised and dissolved directly in spotting solution. The eventual microarray performance was not affected by this simplification. For spotting on maleimide- and succinimidyl-activated surfaces, an aqueous solution of 1 M betaine was used, whose pH was adjusted with NaOH. The main effect of the betaine was a significant reduction in evaporation during the spotting process (20). Since small volumes of one to a few nanolitres were spotted, this significantly increased the period during which a reaction between PNA and surface could take place, resulting in better and more homogeneous binding. For streptavidin-coated slides, 1× BW buffer was used as spotting solution. Here, evaporation was avoided by adjustment of the air humidity during the whole reaction time (spotting process as well as incubation time).

## PNA purification by selective binding of full-length molecules

To avoid time-consuming and expensive purification of the molecules by means such as HPLC, we tested different coupling chemistries for achieving a selective binding of the full-length PNA products to the microarray surface. Eventually, the following combinations were analysed in detail: succinimidyl-activated slides and unmodified oligomers with a primary amino group ($H_2N$-PNA-$CONH_2$; Fig. 3a); maleimide-activated or gold-coated slides and PNA, to which a cysteine was added during the last synthesis cycle (Cys-PNA-$CONH_2$; Fig. 3b); streptavidin-coated slides and PNA with an N-terminal biotin (biotin-PNA-$CONH_2$; Fig. 3c). All shorter PNA derivatives visible in the MALDI spectra, resulting from incomplete condensation during PNA synthesis, were capped prior to the following extension reaction and had therefore acetylated amino ends that should not bind to the microarray surfaces. Thus, spotting and purification could take place concomitantly.

PNA 13mers of different sequence (Table 1) were synthesised. For each sequence, oligomers with all three above-mentioned N-termini were produced. A portion of each PNA oligomer with a terminal amino group was subsequently capped by five successive acetylation reactions and employed as a negative control. Additionally, oligomers of entirely unrelated sequence were produced in order to check for non-specific hybridisation. Two processes were used to monitor the efficiency of the on-chip purification by selective binding (Fig. 3). First, the PNA synthesis products were spotted onto the adequate microarray surfaces and hybridised with complementary Cy3- or Cy5-labelled DNA oligonucleotides (Table 1). The signal intensities obtained at the respective spots were compared to the results at the positions of the fully acetylated PNAs. As a second measure, crude PNA products with an intentionally high amount of incomplete and thus acetylated sequences were attached to slides modified with an acid-labile linker system ($H_2N$-PNA-$CONH_2$ and Cys-PNA-$CONH_2$) or directly to a streptavidin surface (biotin-PNA-$CONH_2$). After washing, the bound PNA molecules were cleaved from the surfaces and analysed by MALDI-TOF mass spectrometry. Only the results for the oligomer *Seq1-PNA* are shown in Figure 3 (second panel), but identical findings were obtained with all other oligomers.

For the succinimidyl-activated microarrays (Fig. 3a), the mass spectrum demonstrates good purification efficiency. The molecular weight of the full-length *Seq1-PNA* (3834.7 Da) increased to 4022.9 Da, since the AEEA-OH linker was still attached to the PNA after release from the support. No peaks from molecules shorter than the original full-length PNA can

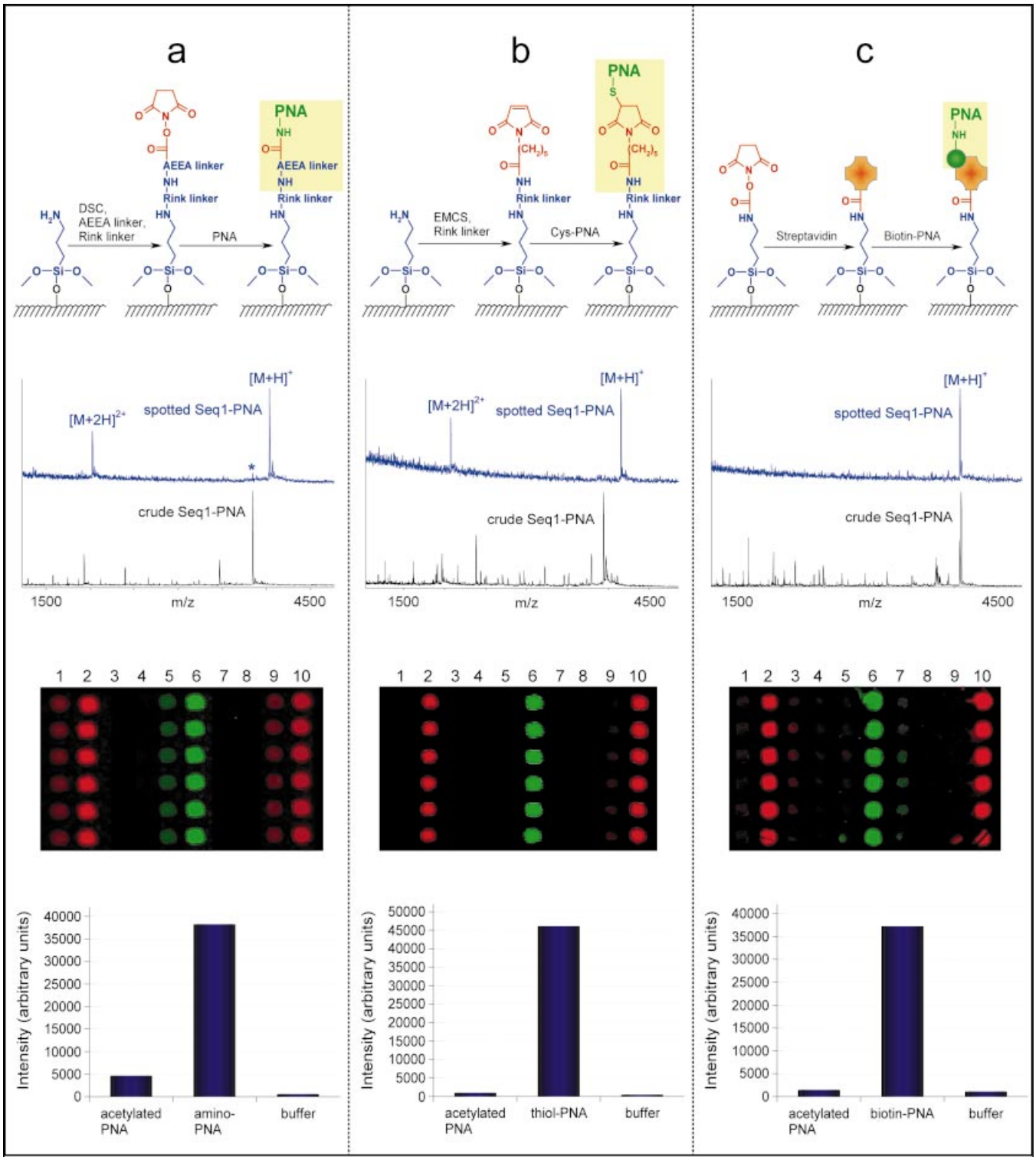


**Figure 3.** Selective binding of full-length PNA molecules. The chemistry that was applied, MALDI-TOF mass spectra of the molecules released from the microarray surface, hybridisation experiments and corresponding signal intensities are shown from top to bottom for the N-terminal (**a**), thiol-modified (**b**) and biotinylated PNAs (**c**). (Top) A scheme of the basic chemistry of attachment is presented. Cleavage releases the kind of molecule highlighted by a yellow background. The MALDI-TOF analyses (second panel) compare spectra of the crude *Seq1-PNA* resulting from synthesis (black lines) with spectra of the same molecule after spotting on a microarray surface and subsequent release (blue lines). The mass difference between the initial full-length PNA peak and the peak of the released molecule matches the mass of the remains of the linker system. The signal marked by an asterisk indicates non-specific interaction. (Third panel) Results obtained after the oligonucleotides *DNA-Seq1-Cy5*, *DNA-Seq2G-Cy3* and *DNA-TC-Cy5* were hybridised at saturating conditions. From left to right, fully acetylated *Seq1-PNA* (lane 1), crude *Seq1-PNA* (lane 2), buffer (lanes 3 and 4), acetylated *Seq2C-PNA* (lane 5), crude *Seq2C-PNA* (lane 6), buffer (lanes 7 and 8), acetylated *TC-PNA* (lane 9) and crude *TC-PNA* (lane 10) were spotted in several copies. (Fourth panel) The actual average signal intensities of the acetylated *Seq2C-PNA*, crude *Seq2C-PNA* and buffer spots are shown.

be seen. Only a small remaining signal was detected at 3834.7 Da itself (marked with an asterisk), indicating the occurrence of some non-covalent interaction of the PNA and surface. A weak signal was also observed on the spots of the fully acetylated PNA in the hybridisation experiments. Non-specific hybridisation of the DNA could be ruled out, since no such effect was seen on unrelated PNA sequences. Initially, we assumed that the acetylation reaction was not quantitative so that remaining primary amino groups could still be available for binding. However, increasing the number of successive acetylation reactions to five did not reduce the observed signal. Covalent binding of acetylated PNAs could have occurred via the less reactive, heterocyclic amino groups of the bases adenine, cytosine and guanine. However, no corresponding peak, which would have a changed molecular mass due to addition of the AEEA-OH linker to the PNA, could be observed by mass spectrometry. Therefore, it is likely that the small portion of non-covalent interaction between the PNA and surface is stable during the hybridisation process and contributes to the signal generated by hybridisation.

Mass spectra of PNAs cleaved from maleimide-activated slides (see, for example, Fig. 3b) show that only completely extended sequences had been attached to the microarray surface. The peaks correspond to the masses of the PNA with maleimide linker group and the amino group of the rink linker ($[M+H]^+$, $m/z$ = 4149.1 Da) as well as the respective doubly charged molecules ($[M+2H]^{2+}$, $m/z$ = 2075.05 Da). The mass introduced by the maleimide linker group is 210.2 Da, which matches the actual mass difference of 210.9 Da found in the spectra. Also, the ratio of the signals at the spots resulting from cysteine-PNAs and the positions of the fully acetylated PNAs were much higher compared to the results on the DSC-activated arrays in the hybridisation experiment.

Efficient on-chip purification could be demonstrated on streptavidin slides (Fig. 3c) as well. No molecule other than full-length PNA was detected by mass spectrometry. Upon hybridisation of DNA samples, the average ratio of the signal intensities of acetylated to biotinylated PNAs fell in between those obtained on succinimidyl- and maleimide-activated slides. However, during the spotting process, biotinylated PNA got stuck to the spotting pins, although extensive washing of the pins between spotting events was performed. Thereby, some carry-over of PNA occurred, eventually causing signals at spots onto which mere buffer samples had been placed. A possibility to circumvent this problem would be the use of non-contact printing devices.

## Evaluation of hybridisation parameters

Generally, the PNA microarrays turned out to be very robust and stable. Arrays on maleimide and succinimidyl ester surfaces especially could be stripped and re-used many times over. No apparent change in signal intensities could be detected after 20 cycles of hybridisation and stripping (data not shown). In order to determine the PNA amount that is optimal for hybridisation, different concentrations of PNA were spotted and hybridised with complementary, fluorescently labelled oligonucleotides (Fig. 4). On DSC-activated slides, an optimal loading was achieved at a PNA concentration between 120 and 160 µM. EMCS-activated surfaces exhibited saturation at ~200 µM. This higher value may be due to the formation of disulfides between PNA molecules with

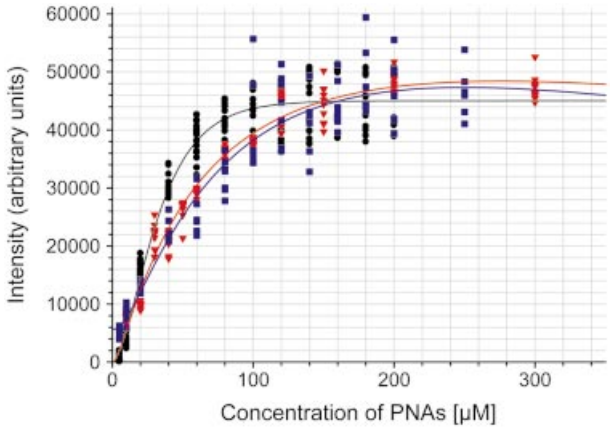


**Figure 4.** Determination of microarray surface loading capacities. Cy5-labelled DNA oligonucleotides were hybridised to microarrays, onto which complementary PNA oligomers had been spotted at concentrations ranging from 5 to 200 µM for DSC slides, 10 to 300 µM for EMCS slides and 5 to 300 µM for strepavidin-coated microarrays. The recorded signal intensities (DSC, black; EMCS, red; strepavidin, blue) indicate the relative binding capacities.

terminal cysteines, resulting in a lower concentration of molecules with a free thiol group. Hydrolysis of *N*-succinimidyl ester groups from the DSC surface may also play a role. On streptavidin surfaces, biotinylated PNAs at concentrations between 160 and 200 µM showed good results. For all three processes, one could expect a reduction in the amount of bound DNA at higher concentrations because of steric reasons. Within the range analysed, however, no such effect was observed.

The selectivity of DNA hybridisation to arrayed PNA molecules had been analysed earlier (8,17). To evaluate the performance of PNA on glass surfaces, the hybridisation of molecules that differ by a single nucleotide was analysed. Figure 5 presents results obtained with the 13mer molecules *Seq2A-PNA*, *Seq2C-PNA*, *Seq2G-PNA* and *Seq2T-PNA* (Table 1). Hybridisation was performed with a mixture of the Cy3-labelled DNA molecule *DNA-Seq2G-Cy3*, which is complementary in sequence to *Seq2C-PNA*, and a Cy5-labelled oligonucleotide that corresponds to bases A, C or T, respectively. In all cases, good discrimination between the four sequence variants was obtained.

## TOF-SIMS detection of hybridisation events

Overall, best results with regard to surface preparation, selective binding of full-length molecules, performance in hybridisation experiments with fluorophor-labelled DNA samples and reusability were obtained by coupling PNAs that carried a thiol group by virtue of a terminal cysteine onto maleimide (EMCS) surfaces. The eventual goal of our study, however, was the production of microarrays to detect hybridisation of unlabelled DNA by TOF-SIMS. For an examination of their performance under such conditions, we spotted four thiol-modified PNAs (*TC-PNA*, *Seq1-PNA*, *Seq2C-PNA* and *Seq3-PNA*; Table 1) in 10 replicates with spot diameters of 100 µm on EMCS-activated silicon wafers. One microarray was hybridised with three Cy5- or Cy3-labelled oligonucleotides, the other incubated with three unlabelled 50mer oligonucleotides (*DNA-TC-long*, *DNA-Seq1-long* and *DNA-*

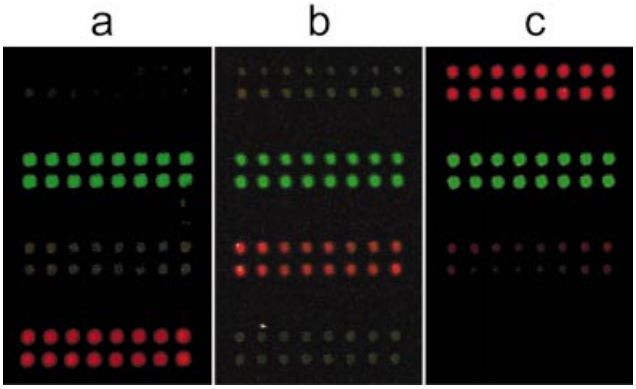


**Figure 5.** Detection of single base mismatches. The 13mer *Seq2-PNA* molecules (Table 1) were spotted on DSC-activated silicon wafers in two rows of eight replicate spots each, separated by two rows of buffer spots. The PNAs were identical in sequence but for the central base, which was (from top to bottom) A, C, G and T, respectively. Slides were hybridised with a mixture of two complementary oligonucleotides: (**a**) *DNA-Seq2G-Cy3* and *DNA-Seq2A-Cy5*; (**b**) *DNA-Seq2G-Cy3* and *DNA-Seq2C-Cy5*; (**c**) *DNA-Seq2G-Cy3* and *DNA-Seq2T-Cy5*.

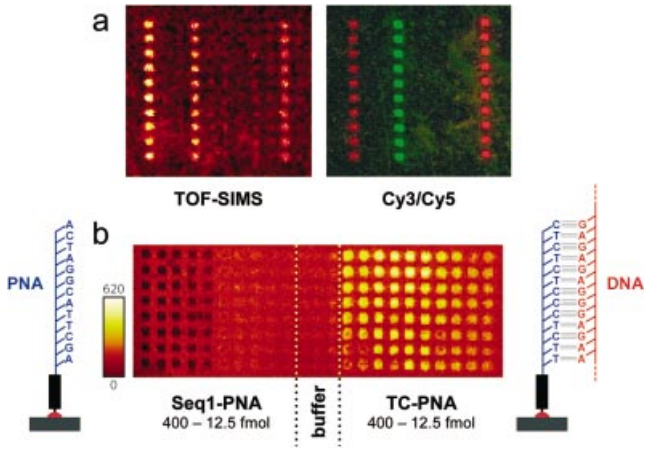


**Figure 6.** Detection of hybridisation of unlabelled DNA. (**a**) From left to right, the following molecules were spotted on maleimide silicon wafers, arranged in columns of 10 replicates each: *Seq1-PNA*, buffer, buffer, *Seq2C-PNA*, buffer, buffer, *Seq3-PNA*, buffer, buffer, *TC-PNA*. Hybridisation was either with a mixture of *DNA-Seq1-Cy5*, *DNA-Seq2G-Cy3* and *DNA-TC-Cy5* (right panel) or the corresponding, unlabelled 50mer oligonucleotides (Table 1) (left panel). The latter experiment was analysed by TOF-SIMS, the results of which are shown in a false colour representation. A short bar indicates the colour code for the signal intensities. (**b**) A dilution series of *Seq1-PNA* and *TC-PNA* was spotted left to right (400, 350, 300, 250, 200, 150, 100, 50, 25 and 12.5 fmol; spot diameter 300 µm) in columns of eight copies on a silicon wafer with a gold surface. Hybridisation was with an unlabelled oligonucleotide that was complementary to *TC-PNA* and analysis by TOF-SIMS. The signal intensities determined at a mass of 79 Da ($PO_3^-$) are presented.

*Seq2G-long*; Table 1) that were complementary to three of the four PNAs. While the fluorescence signals were detected by a fluorescence scanner, TOF-SIMS was employed for the detection of hybridisation of the unlabelled DNA. Both experiments produced identical results. Signal was obtained only on a PNA that was complementary to one of the DNA samples (Fig. 6a).

In other experiments, we spotted dilutions of thiol-modified PNAs onto silicon wafers with gold surfaces. A typical result obtained with *TC-PNA* and *Seq1-PNA* (from left to right, 400 down to 12.5 fmol; spot diameters 300 µm) is presented in Figure 6b. This microarray was hybridised with an unlabelled oligonucleotide that was complementary only to *TC-PNA* and analysed by TOF-SIMS. A strong signal was detected even on PNA of low concentration. Although no optimisation with regard to the absence of phosphate contamination had been performed, there was only a relatively low background signal around spots or on spots of non-complementary PNA sequence. As a matter of fact, presence of high concentrations of unrelated PNA suppressed the background signal significantly. TOF-SIMS measurements analyse only the upmost monolayer of molecules. Therefore, the primary ion beam is prevented by the layer of PNA oligomers from penetrating the microarray surface. While the surface is slightly contaminated with phosphates, the PNA layer does not contain any of them, thus no background signal is produced.

The images shown were produced by scanning the entire surface pixel by pixel. Since molecules are removed by TOF-SIMS, the microarrays were not reused in such cases. However, scanning was done in order to produce complete images. This is not necessarily required in real analyses. Only a small portion of each spot could be analysed, leaving the remaining area unaffected and thereby available to subsequent analyses.

## DISCUSSION

The ability to purify crude PNA products by an on-chip purification process that is intrinsic to the application of the PNA to the microarray surface provides the means to produce complex PNA microarrays of high oligomer quality. The automated PNA synthesis in 96-well plates permits low cost production of PNA in quantities that suffice for the production of very many microarray copies. Beside this application, the PNA molecules can also be used for other purposes, such as fluorescence *in situ* hybridisation experiments (21,22), as capture probes for RNA purification (23), as molecular beacons (24) or as PNA openers (25,26), for example. Concerning microarray production, we established three purification schemes that produced good results, crucial to performing analyses such as the detection of SNPs accurately, for which purity of the PNA molecules and a low background signal are a prerequisite. Maleimide surfaces in combination with thiol-modified PNAs were the overall best choice. It should be noted that in all experiments we compared hybridisation signals on identical concentrations of fully acetylated PNA and normal PNA products. The absolute amount of shorter than full-length, and thus acetylated, derivatives in a normal PNA synthesis is much smaller than the quantity of acetylated PNA used in our experiments. Therefore, the hybridisation signal that results from non-specific binding of such molecules is significantly lower than what was measured in our set-up.

In addition to analyses with fluorescently labelled samples, the microarrays permitted specific and rather sensitive detection of unlabelled DNA targets by TOF-SIMS. Gold-coated and maleimide-activated silicon wafers in combination with thiol-modified PNAs proved especially promising in this respect. Even with an entirely unrefined system, sensitivity

was good, the detection limit being in the attomolar range. With the potential of hybridising long DNA fragments, optimisation of the phosphate background in the buffer and surface coatings, as well as significant reductions in spot size, the last also adding a positive effect on sensitivity that is predicted by the ambient analyte theory (27), much better sensitivity is to be expected. The detection mode could also be improved by various means (Feldner *et al*., in preparation). Combined, these measures might pave the way for an analysis of complex DNA samples without the need for prior amplification and labelling. Rather than being restricted by the sensitivity of detection, the ability to select oligomers of unique sequence might become the limiting factor. At least in some assays, such as looking for infections by searching for contaminating DNA from other organisms, this could be overcome because of differences in sequence composition. However, single base mismatches could also be very well discriminated with 16mer PNA oligomers (see, for example, 8). Thus, oligomer length could be adapted to the complexity of the sample.

## ACKNOWLEDGEMENT

This work was financially supported by the German Federal Ministry of Education and Research (BMBF).

## REFERENCES

1. Schena,M., Shalon,D., Davis,R.W. and Brown,P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, **270**, 467–470.
2. Guo,Z., Guilfoyle,R.A., Thiel,A.J., Wang,R. and Smith,L.M. (1994) Direct fluorescence analysis of genetic polymorphisms by hybridisation with oligonucleotide arrays on glass supports. *Nucleic Acids Res.*, **22**, 5456–5465.
3. Dawson,E., Abecasis,G.R., Bumpstead,S., Chen,Y., Hunt,S., Beare,D.M., Pabial,J., Dibling,T., Tinsley,E., Kirby,S. *et al*. (2002) A first-generation linkage disequilibrium map of human chromosome 22. *Nature*, **418**, 544–548.
4. Adorjan,P., Distler,J., Lipscher,E., Model,F., Müller,J., Pelet,C., Braun,A., Florl,A.R., Gutig,D., Grabs,G. *et al*. (2002) Tumour class prediction and discovery by microarray-based DNA methylation analysis. *Nucleic Acids Res.*, **30**, e21.
5. Dubiley,S., Kirillov,E. and Mirzabekov,A. (1999) Polymorphism analysis and gene detection by minisequencing on an array of gel-immobilized primers. *Nucleic Acids Res.*, **27**, e19.
6. Syvänen,A.-C. (1999) From gels to chips: "minisequencing" primer extension for analysis of point mutations and single nucleotide polymorphisms. *Hum. Mutat.*, **13**, 1–10.
7. Dumur,C.I., Dechsukhum,C., Ware,J.L., Cofield,S.S., Best,A.M., Wilkinson,D.S., Garrett,C.T. and Ferreira-Gonzalez,A. (2003) Genome-wide detection of LOH in prostate cancer using human SNP microarray technology. *Genomics*, **81**, 260–269.
8. Weiler,J., Gausepohl,H., Hauser,N., Jensen,O.N. and Hoheisel,J.D. (1997) Hybridisation based DNA screening on peptide nucleic acid (PNA) oligonucleotide arrays. *Nucleic Acids Res.*, **25**, 2792–2799.
9. Nielsen,P.E., Egholm,M., Berg,R.H. and Buchardt,O. (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science*, **254**, 1497–1500.
10. 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) PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature*, **365**, 566–568.
11. Demidov,V.V., Potaman,V.N., Frank-Kamenetskii,M.D., Egholm,M., Buchard,O., Sonnichsen,S.H. and Nielsen,P.E. (1994) Stability of peptide nucleic acids in human serum and cellular extracts. *Biochem. Pharmacol.*, **48**, 1310–1313.
12. Wittung,P., Nielsen,P.E., Buchardt,O., Egholm,M. and Norden,B. (1994) DNA-like double helix formed by peptide nucleic acid. *Nature*, **368**, 561–563.
13. Ray,A. and Norden,B. (2000) Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. *FASEB J.*, **14**, 1041–1060.
14. Arlinghaus,H.F., Kwoka,M.N. and Jacobson,K.B. (1997) Analysis of biosensor chips for identification of nucleic acids. *Anal. Chem.*, **69**, 3747–3753.
15. Arlinghaus,H.F. Ostrop,M., Friedrichs,O. and Feldner,J. (2003) Genome diagnostic with TOF-SIMS. *Appl. Surf. Sci.*, **203/204**, 689–692.
16. Matysiak,S., Würtz,S., Hauser,N.C., Gausepohl,H. and Hoheisel,J.D. (1999) PNA-arrays for nucleic acid detection. In Nielsen,P. and Egholm,M. (eds), *Peptide Nucleic Acids: Protocols and Applications*. Horizon Scientific Press, Wymondham, UK, pp. 119–128.
17. Matysiak,S., Reuthner,F. and Hoheisel,J.D. (2001) Automating parallel peptide-synthesis for the production of PNA-library arrays. *Biotechniques*, **31**, 896–904.
18. Frank,R. (1992) Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron*, **48**, 9217–9232.
19. Awasthi,S.K. and Nielsen,P.E. (2002) Parallel synthesis of PNA-peptide conjugate libraries. *Comb. Chem. High Throughput Screen*, **5**, 253–259.
20. Diehl,F., Grahlmann,S., Beier,M. and Hoheisel,J.D. (2001) Manufacturing DNA-microarrays of high spot homogeneity and reduced background signal. *Nucleic Acids Res.*, **29**, e38.
21. Rigby,S., Procop,G.W., Haase,G., Wilson,D., Hall,G., Kurtzman,C., Oliveira,K., Von Oy,S., Hyldig-Nielsen,J.J., Coull,J. *et al*. (2002) Fluorescence in situ hybridization with peptide nucleic acid probes for rapid identification of *Candida albicans* directly from blood culture bottles. *J. Clin. Microbiol.*, **40**, 2182–2186.
22. Larsen,R.D., Schonau,A., Thisted,M., Petersen,K.H., Lohse,J., Christensen,B., Philip,J. and Pluzek,K.J. (2003) Detection of gamma-globin mRNA in fetal nucleated red blood cells by PNA fluorescence *in situ* hybridisation. *Prenat. Diagn.*, **23**, 52–59.
23. Chandler,D.P. and Jarrell,A.E. (2003) Enhanced nucleic acid capture and flow cytometry detection with peptide nucleic acid probes and tunable-surface microparticles. *Anal. Biochem.*, **312**, 182–190.
24. Kuhn,H., Demidov,V.V., Coull,J.M., Fiandaca,M.J., Gildea,B.D., Frank-Kamenetskii,M.D. (2002) Hybridisation of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. *J. Am. Chem. Soc.*, **124**, 1097–1103.
25. Demidov,V.V. and Frank-Kamenetskii,M.D. (2002) PNA openers and their applications. *Methods Mol. Biol.*, **208**, 119–130.
26. Kuhn,H., Hu,Y., Frank-Kamenetskii,M.D. and Demidov,V.V. (2003) Artificial site-specific DNA-nicking system based on common restriction enzymes assisted by PNA openers. *Biochemistry*, **42**, 4985–4992.
27. Ekins,R., Chu,F. and Biggart,E. (1990) Multispot, multianalyte, immunoassay. *Ann. Biol. Clin.*, **48**, 655–666.