Development towards label- and amplification-free genotyping of genomic DNA

O. Brandt[a], J. Feldner[b], S. Hellweg[b], M. Schröder[b], A. Stephan[a], H.F. Arlinghaus[b], J.D. Hoheisel[a], A. Jacob[a]*

[a]*Functional Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany*

[b]*Physikalisches Institut, Universität Münster, Wilhelm-Klemm-Strasse 10, 48149 Münster, Germany*

* Corresponding author. Tel.: +49-6221-42-4685; fax: +49-6221-42-4687.
E-mail address: a.jacob@dkfz.de (A. Jacob).

Abstract

Peptide nucleic acid (PNA) microarrays have been used for the analysis of unlabelled DNA molecules by time-of-flight secondary mass spectrometry (ToF-SIMS). PNAs were synthesised with an automated system in multi-well plates followed by the spotting full length molecules only onto derivatised silicon surfaces using a common split-pin contact printing instrument. After DNA hybridisation, phosphate specific signals were detected and visualised by ToF-SIMS imaging. Because phosphorus is entirely missing in PNAs but is an integral part of nucleic acids, specific signals were only detected on spots at which DNA bound to complementary PNA probes. Combining PNA microarrays with ToF-SIMS detection enables the sensitive analysis of DNA or RNA targets without the need for introducing labels like fluorescent groups.

Keywords: PNA; ToF-SIMS; Label-free detection; Microarray

1. Introduction

Microarray technology is a well established method for the analysis of complex probes in a variety of biological research areas, including transcriptional profiling [1], single nucleotide polymorphism (SNP) analysis [2], methylation studies [3] or the detection of bacterial contaminations and infections [4]. In general, microarrays consist of solid supports like glass or silicon slides containing an array of small spots with diameters in the micrometer range of well defined DNA or PNA sequences (probes). These probes are capable to bind complementary DNA or RNA molecules (targets). After successful hybridisation experiments, the target molecules are detected via a label. Usually this is done by fluorescence detection, which explains the need for an additional labelling step of the RNA or DNA target prior to hybridisation. Various efforts have been made for a label-free analysis to circumvent this extra step. These are surface plasmon resonance [5], electronic [6], electrochemical [7] and ToF-SIMS detection systems [8], besides others. The different backbones of DNA and PNA open up the way for ToF-SIMS as a means for detection, a sensitive method for molecular ion detection in the lower mass range. ToF-SIMS imaging of a microarray surface to which PNA probe molecules are covalently attached at defined positions allows the visualisation of the PO_2^- and PO_3^- secondary ions that originate only from the backbone of DNA or RNA molecules after hybridisation to the appropriate probe molecule. On spots that contain PNA only, no phosphate specific signals can be detected.

PNA was introduced by Nielsen et al. [9] and is a synthetic DNA mimic with a peptide like backbone consisting of N-(2-aminoethyl)-glycine units to which the nucleobases are bound via methylene carbonyl linker (Fig. 1). This chemical composition enables the synthesis of PNAs using common peptide chemistry and, more importantly, allows a strong hybridisation to complementary DNA and RNA molecules. The neutral backbone of PNA increases the thermal stability of a PNA:DNA duplex in comparison to the respective DNA:DNA duplex of the same sequence [10, 11]. Particularly when salt concentrations are low, this effect is stronger because of the lack of positive ions that counteract the repulsion of two negatively charged backbones in DNA:DNA duplexes. This effect is not important for PNA:DNA duplexes because of the uncharged nature of PNA. Hybridisation buffers with a low salt content minimise secondary structures in DNA or RNA molecules that result from self-complementary sequences, making the DNA or RNA more accessible to PNA molecules targeting these regions.

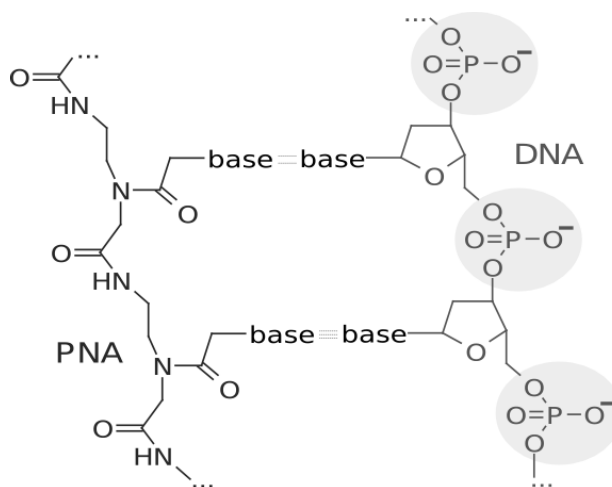

Fig. 1. PNA:DNA duplex with characteristic backbones.

2. Experimental

2.1. PNA synthesis

PNA-synthesis took place in either 96- or 384-well microtiter plates in a fully automated system [12]. PNAs are synthesised in a similar way common peptide synthesis is performed, whereas the growing chain is elongated by a monomer protected with an N-terminal Fmoc and an additional side-chain protection group. After coupling is completed, the terminal Fmoc-protection group is removed and subsequent cycles are performed until the desired molecule is assembled.

The PNA molecules were synthesised on Fmoc-protected Rink resin LS or on a TentaGel-S RAM Fmoc resin with a substitution of about 0.2 mmol/g. After swelling the resin for 1 h in N,N'-dimethylformamide (DMF), 2 mg were distributed to each well of the synthesis plate. The coupling cycle was started with the cleavage of the Fmoc protection groups. This was done by two successive incubations (1 and 5 min) with 30 µl 20% piperidine in DMF. The Fmoc groups and piperidine were washed away five times with 80 µl DMF followed by a double coupling (2x 20 min) of the first monomer. Each of the two coupling solutions consisted of 4 µl Fmoc-PNA-monomer (0.3 M in 1-methyl-2-pyrrolidone (NMP), 2 µl O-(7-azabenzotriazol-1-yl)-N,N,N'N'-tetramethyluronium hexafluorophosphate (HATU) (0.6 M in DMF) and 2 µl base mix (0.6 M diisopropylethylamine (DIPEA) and 0.9 M 2,6-lutidine in DMF). After coupling the resin was washed three times with 80 µl DMF. Not elongated sequences were acetylated by incubation in 30 µl capping mix (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. The cycle was repeated until the synthesis of the desired PNA sequence was completed. Finally the terminal Fmoc group was removed as described above. The resin was washed another five times with 80 µl DMF and three times with 80 µl 1,2-dichloroethane. After synthesis, the final PNA molecules were cleaved from the dried resin by incubation in 150 µl 95% trifluoroacetic acid (TFA) and 5% triisopropylsilane (TIS) for 1.5 h. After elution of the molecules with another 150 µl cleavage mix, precipitation of PNAs was done by adding 1 ml of ice-cold ether. PNAs were washed another time with ether, dried and dissolved in 100 µl water. Quality control of all synthesised PNAs was done by MALDI-TOF mass spectrometry.

2.2. Microarray surface preparation

For the microarrays, oxidised silicon wafers were silanised to add functional groups. The silicon surfaces were cleaned with dimethyl sulfoxide, ethanol and water. After etching for 1 h and additional 15 min in an ultrasonic bath, the wafers were washed with water and ethanol. Silanisation was done by shaking the slides in a solution of 1 ml [3-aminopropyl]triethoxysilane in 20 ml 95% ethanol for 1 h, followed by sonication for 15 min. The wafers were then washed in ethanol, water, dried under nitrogen and baked at 110°C for 20 min. Depending on the coupling method of the PNAs, the surfaces were further modified with succinimidyl ester or maleimide groups (see below).

2.2.1. Succinimidyl ester activation

Prior to the spotting of PNAs with N-terminal amino group, the silanised 2x2 cm^2 silicon wafers were activated by incubation in a freshly prepared solution of 150 mg N,N'-disuccinimidyl carbonate (DSC) and 0.5 ml DIPEA in 14.5 ml dried acetone for 2 h. After washing twice with acetone and dichloroethane, the dried wafers were immediately taken for the spotting procedure.

2.2.2. Maleimide activation

For PNAs with a thiol group at their N-terminus, silanised wafers were activated directly before the spotting process with a solution of 20 mM N-hydroxysuccinimide ester (EMCS) in DMF containing 10% diisopropylethylamine. The 2x2 cm^2 wafers were activated with 40 µl of this solution under a coverslip for 3 h at room temperature, washed twice with DMF and once with acetone, air dried and directly taken for the spotting of PNAs.

2.3. PNA spotting

PNAs with free amino group at their N-terminus were spotted onto succinimidyl ester activated slides whereas maleimide or gold surfaces were used for PNAs with N-terminal thiol group. For the former process, PNAs was diluted to 200 µM in water with 1 M betaine and a pH of 7.5. In the latter case, 200 µM PNA solutions in water with 1 M betaine and a pH of 7.0 were used.

The spotting was performed with an SDDC-2 DNA Micro-Arrayer from Engineering Services Inc. (Toronto, Canada) and SMP3 pins (TeleChem International, CA, USA). The PNA solutions were distributed onto the surfaces with spot to spot distances of 170 µm. After spotting, the wafers were incubated overnight at room temperature followed by deactivation of the surface. The DSC surfaces were blocked for 2 h in a solution of 50 mM succinic anhydride and 150 mM 1-methylimidazole in dichloroethane, washed twice with dichloroethane and dried with nitrogen. The maleimide and gold slides were deactivated in 1 mM 3-mercaptopropionic acid in ethanol for 30 min, washed three times with ethanol, DMF and ethanol again.

All surfaces were then rinsed twice with hot buffer (5 mM sodium phosphate, 0.1% SDS), incubated in hot water for 10 min, followed by rinsing with 1 M NaCl in 0,1% aq. TFA and water. After drying with nitrogen the slides were ready for hybridisation experiments.

2.4. Microarray hybridisation

Different unlabelled DNA oligonucleotides were mixed to a final concentration of 0.2 M in 0.1x SSarc buffer (60 mM sodium chloride, 6 mM sodium citrate, 0.72% v/v N-lauroylsarcosine sodium salt solution). The 2x2 cm^2 PNA microarrays were incubated with 20 µl of this solution under a cover slip at 38°C for 2 h in a hybridisation chamber (TeleChem). Finally the slides were washed two times with 0.1x SSarc, one time with water and dried with nitrogen.

2.5. ToF-SIMS analysis

ToF-SIMS imaging of the PNA microarrays was performed with a ToF-SIMS type IV instrument. A primary ion beam of Ar^+ or Xe^+ with energies of 10 keV and an ion dose of about 10^{12} cm^{-2} was used to scan the 2 x 2 cm chip surface. For this purpose the sample stage was moved in x,y-direction under the ion focus. The resulting mass spectra enabled the visualisation of molecular distribution for PO_2^- (63 amu) and PO_3^- (79 amu) secondary ions.

3. Results and discussion

PNA synthesis was performed in microtiter plates with optimised washing, coupling and capping steps. Except for the final cleavage of PNAs from the resin, all reagents and washing solutions were distributed by a robotic system fully automated and computer controlled. A vacuum manifold extracted the solutions into the waste container. Important factors for successful synthesis were intensive washing and double couplings with the activation reagent HATU, which is more reactive compared to HBTU for example. Besides this, the low resin loading with functional amino groups is also a critical point. High loading capacities reduces the quality of PNA synthesis because more truncated sequences are formed due to sterical reasons. Taking these factors into account, molecules with a length of more than 20 PNA monomers, modified with additional linker, amino acids or other terminal groups like biotin, could easily be assembled (Fig. 2). The synthesis was carried out in a 400 nmol scale in 384- or 96-well plates. This scale is sufficient for the spotting of thousands of microarrays. However it is possible to increase or decrease the scale for other applications as well by using larger quantities of synthesis resin and reagents in 96-well plates or in little columns with frits.

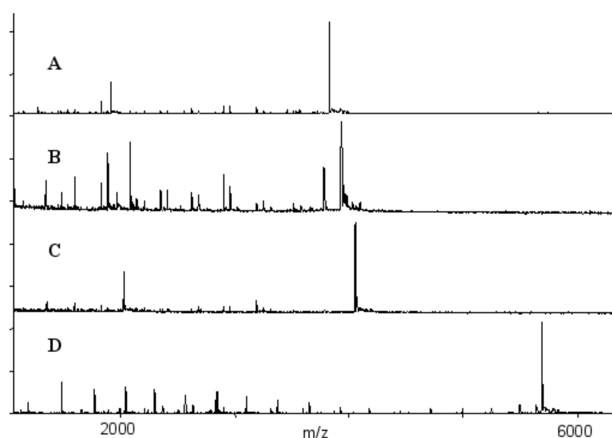

Fig. 2. MALDI-ToF-MS spectra of PNAs synthesised in microtiter plates. Following PNAs are shown: Cysteine-Li-TTGAATCGCTCGA-Li-$CONH_2$ (A), Li- TTGAATCGCTCGA-Li-$CONH_2$ (B), Biotin-Li-TTGAATCGCTCGA-Li-$CONH_2$ (C) and Li-Li-CCATACAAATTTCAGGATTT (D).

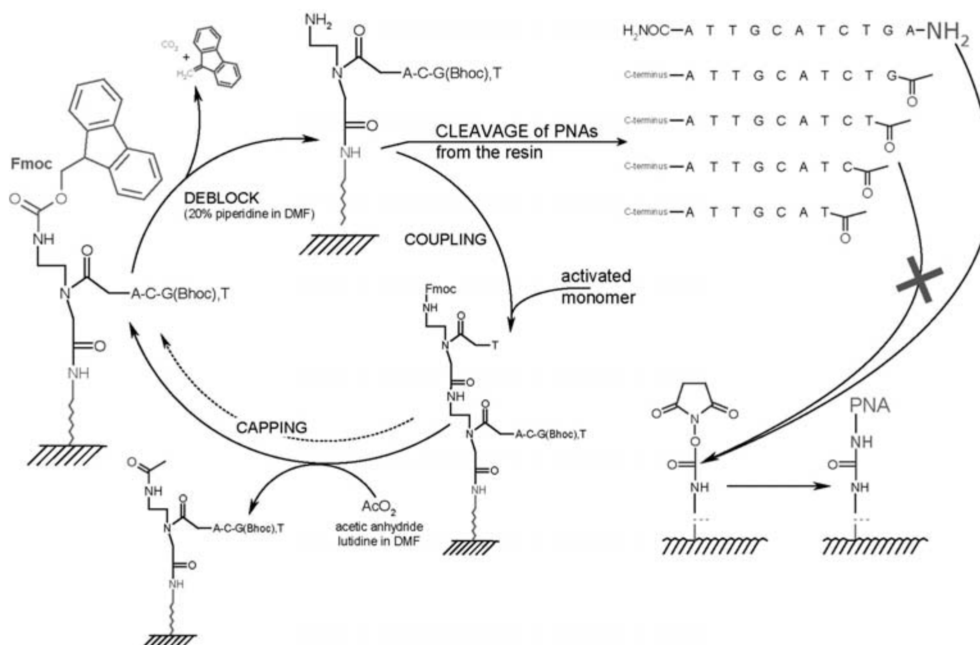

Fig. 3. PNA synthesis cycle. Coupling of un-purified PNA after synthesis is shown on a N,N'-disuccinimidyl carbonate (DSC) activated surface.

The covalent binding of PNAs to maleimide activated or gold-coated microarrays was done with molecules containing cysteine at their N-terminal ends, while for the spotting onto succinimidyl ester surfaces PNAs with usual N-terminal amino function were used. For both coupling methods, an on-chip purification of full-length PNAs was achieved from truncated sequences that are formed during the synthesis due to selective binding of the N-terminus to the respective groups at the activated surfaces. All shorter sequences, resulting from incomplete condensation during the synthesis cycles, were capped at their N-terminus by acetic anhydride. The capping step modifies the not elongated sequences with an acetyl group that can not be extended in the following cycles and should not bind to the activated surfaces (Fig. 3). Through this, a time-consuming and expensive HPLC purification prior to the spotting could be avoided [12]. Typical results of the purification process on the chip surfaces are shown in Figure 4.

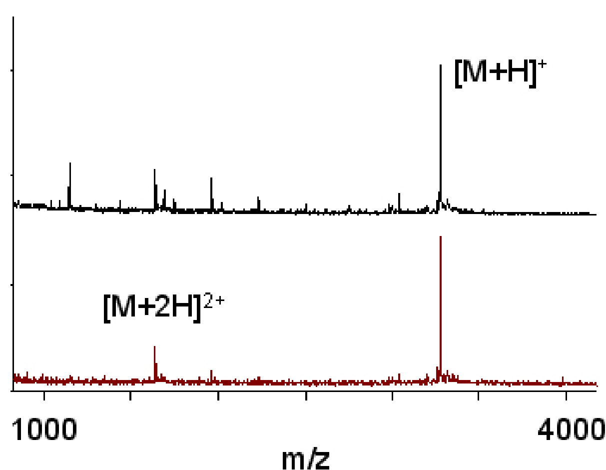

Fig. 4. Efficiency of the on-chip purification. MALDI-TOF spectra of an example for a crude PNA (top) and the same PNA, which was spotted, washed and cleaved from the chip surface (bottom).

To verify the efficiency of the on-chip purification, modified surfaces with a cleavable linker system and an additional mass-tag were used. After spotting of crude PNAs, washing of the arrays was optimised by monitoring the PNA products cleaved from the surfaces. Covalently bound PNAs could be identified by the additional mass-tag. With optimised washing procedure no signals for by-products or unspecifically bound PNAs were observed in the mass spectra. Only the singly ($[M+H]^+$) and double charged ($[M+2H]^{2+}$) signals of the mass-tagged full-length PNA were detected.

The selectivity of PNAs in chip based assays has been demonstrated in several assays [e.g., 13, 14]. Overall, selectivity is similar to what can be achieved with DNA probes. Discrimination is best, if the mismatch is located in a central position of the oligomer. Problems regarding the non-specific binding of DNA to PNA that would result in false positive readings are the same irrspective of the detection mode. Dependent on the particular application, an optimised set of probes and adjusted hybridisation conditions (e.g. buffer and temperature) had to be selected.

To compare fluorescence detection with ToF-SIMS imaging, four different PNAs with N-terminal cysteine were spotted in replicates onto maleimide activated silicon slides. The size of the spots was about 100 µm in diameter. One array was hybridised with unlabelled, the other with Cy3- and Cy5-labelled oligonucleotides that were complementary to three of the four PNAs on the chip. For both, the fluorescence detection with a common scanner and the ToF-SIMS imaging of PO_3^- ions, signals were only obtained on PNA spots to which the hybridised DNA molecules were complementary (Fig. 5). No unspecific hybridisation of PNA_4 was observed. Comparing the ToF-SIMS and the fluorescence images, differences in the signal intensity ratios of PNA_2 and PNA_3 can be observed. One reason for this effect is the use of two different dyes (Cy3 and Cy5). Fluorescence intensities varied due to differences in the laser excitation and quantum yields. However, the inverse signal intensity ratios of PNA_2 and PNA_1 in fluorescence and ToF-SIMS detection cannot be explained by this. It could be that the fluorophor is influencing the hybridisation behaviour, thus causing the difference compared to the unlabelled molecules used for ToF-SIMS detection. For microarray applications like the identification of bacteria or the analysis of SNPs, however, usually no quantification is required. Additional experiments with both synthetic DNA-oligonucleotides as well as RNA samples showed the possibility of ToF-SIMS to detect phosphate specific signals from DNA in the attomole range (data not shown).

Name	**Sequence**
PNA_1	X-Li-TTCTCCCTCTCTC-Li-$CONH_2$
PNA_2	X-Li-AGCTTACGGATCA-Li-$CONH_2$
PNA_3	X-Li-TTGAATCGCTCGA-Li-$CONH_2$
PNA_4	X-Li-TAAGTCTTAGTCATT-Li-$CONH_2$
PNA_5	H_2N-TTTAGGGGTGAC-$CONH_2$
PNA_6	H_2N-TTTAGAGGTGAC-$CONH_2$
PNA_7	H_2N-CTTTAGAGGTGA-$CONH_2$
PNA_8	H_2N-CTTTAGAGGTGA-$CONH_2$
DNA_1	5'-TTCGATAGCAGCTAAATTCTGAGAGAGGGAGAATTCTACAATAACTGCGC-3'
DNA_2	5'-AGCGATAGCAGTTTGACCATCTGATCCGTAAGCTGCGTAAGCCGTATTCC-3'
DNA_3	5'-GCTTTACCATTGATACTCTTCGAGCGATTCAATCGGTCAGAATACTTCTAC-3'

Tab. 1. PNA and DNA sequences. X = cysteine or H_2N at the N-terminus. Li = AEEA-OH linker.

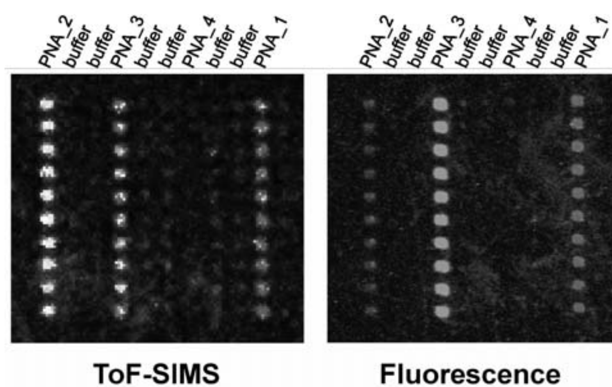

Fig. 5. Comparision of ToF-SIMS and fluorescence detection. Following molecules were spotted in columns of 10 replicates from left to right onto maleimide slides: PNA_2, buffer, buffer, PNA_3, buffer, buffer, PNA_4, buffer, buffer, PNA_1. Hybridisation of the slide for ToF-SIMS detection was done with three unlabelled DNA oligomers (DNA_1, DNA_2 and DNA_3) while for the fluorescence detection corresponding Cy3- and Cy5-labelled oligonucleotides were used. The results for PO_3^- and fluorescence intensities are shown.

To date, ToF-SIMS detection is not a proper competition for the established optical detection systems. The overall speed of detection is far inferior, if the entire microarray needs to be scanned. However, in the anticipated system, the spots are much smaller than in the current layout and their exact location is known. Then it is not required to analyse the entire spot, let alone the space in between spots. For its high sensitivity, only a small area of the respective PNA-feature would be analysed for the presence of phosphate ions, thus potentially even surpassing the speed of optical detection. Also in terms of cost, PNA microarrays are not yet competitive. The reason for this, however, is the high cost of the PNA-monomers. This is due to the relatively low consumption of PNA-monomers as a whole, since the basic components and the production process as such are not that different in cost compared to the production of DNA-monomers. If more material would be consumed, prices could well be in the range of DNA-monomers.

Using primary ions other than Argon, higher signal intensities can be achieved. Therefore, an improvement in sensitivity beyond the attomole detection limit reported so far can be achieved. Initial results with gold and gold-cluster ions (not presented) indicated that even a few molecules could be possible. Then, the amplification of the target sequence prior to analysis would not be necessary anymore, which is the objective of a project pursued in an ongoing collaboration with three company partners.

Quantification would be difficult to be implemented, although not impossible at all. Currently, however, the objective is the establishment of a system for the identification of single nucleotide polymorphisms, may they be naturally occurring or be introduced artificially for the identification of methylation differences in genomic DNA [15].

4. Conclusion

The automated PNA synthesis in 384- or 96-well plates enables the production of PNA microarrays at relatively low costs. The concomitant spotting and purification of PNAs on the basis of site-directed coupling to activated surfaces results in PNA chips with high oligomer quality. The data presented shows that PNA arrays are appropriate tools for SNP detection without any labelling of the analysed samples (or probes) prior to hybridisation. Furthermore ToF-SIMS and fluorescence analysis data are comparable. Even though some work has to be done, PNA arrays in combination with ToF-SIMS imaging show a high potential as a sensitive method for label-free DNA diagnostic on a chip. For its sensitivity, even the analysis of unamplified material should be possible.

Acknowledgements

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