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Production of PNA-Arrays for Nucleic Acid Detection

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Abstract

In recent years, the use of microarray technologies for the analysis of nucleic acids has become routine, and much more is still to come. Because of the special features of PNA-DNA interaction, the use of PNA-oligomers as chip-bound receptors could be advantageous for some applications. In this chapter, procedures for the production of PNA-microarrays are presented. Arrays were produced either by direct *in situ* synthesis or by a two-step process that consists of a highly parallel, fully automated synthesis of PNA-molecules and their subsequent attachment to the microarray-surface. Currently, the latter technique permits higher densities and allows for sample purification during the spotting. Synthesis occurs in resin-filled micro-well plates followed by a release from the support and spotting of the crude products to glass slides or silicon wafers. Selective binding via the amino-ends provides a means for a simple and effective purification of the synthesis products. Only full-length molecules are bound

resulting in high-quality microarrays. Here, detailed synthesis protocols based on Fmoc chemistry are presented for both the *in situ* and the micro-well synthesis technique. MALDI-TOF mass spectrometry and HPLC were applied for quality control. The utility of the different procedures and the influence of parameters that are critical for production and application of PNA-microarrays are discussed.

Background

Microarray-based hybridisation analysis has become a powerful tool in biological and biomedical research offering the means for rapid and cost-effective screens. The technology is frequently used for studying variations in transcript levels, mutational analysis and the investigation of single nucleotide polymorphisms (SNPs), for example. However, especially the last kind of analysis still lacks the throughput required for a population-wide assessment of genetic risk factors. Particularly to this end, microarrays made of PNA-oligomers instead of DNA-oligonucleotides could be superior in many respects (1), since PNA exhibits several advantageous features. PNA-DNA interactions are stronger than the stability of the respective DNA-DNA or DNA-RNA duplexes. Because of the PNA's neutral backbone, there is no repulsion of the negatively charged nucleic acid. Hybridisations can therefore be carried out at low-salt or even no-salt conditions, resulting in a better accessibility of the target-DNA due to fewer secondary structures. The high enzymatic and chemical resistance of PNA leads to stable and robust microarrays, which can be stored for a long time and re-used several times without a loss in performance. Furthermore, the chemical difference between DNA and PNA – the PNA lacking phosphate groups in its backbone – permits an alternative mode of detection (2). PNA-DNA or PNA-RNA duplexes can be visualised by secondary ion mass spectrometry (SIMS), using as label the phosphates that are integral part of the nucleic acids, while missing entirely in PNA. Because of the method's sensitivity, a direct analysis of DNA or RNA-samples without any prior amplification or labelling is well within reach.

Table 1. Reaction conditions for PNA synthesis cycles.

	Synthesis in microtitre well plates	***In situ* synthesis**
Solid support	Rink resin LS	Aminated polypropylene or cellulose membranes
Coupling	To each well: 8 µl of activated monomer solution: 4 µl Fmoc monomer (0.3 M in NMP), 2 µl HATU (0.54 M in DMF), 2 µl base mix (0.6 M diisopropylamine, 0.9 M 2,6-lutidine in DMF) preactivation time: 1 min coupling time: 20 min 2 subsequent couplings, with a DMF washing step in between	To each spot: ~ 0.2 µl activated monomer solution: 1:1:1 mixture of the following solutions: 0.3 M Fmoc-monomer, 0.3 M HOAt and 0.4 M DIC, each in NMP preactivation time: 1 min coupling time: > 15 min 3 subsequent couplings
Washing	3x with 80µl DMF	----------
Capping	5% acetic anhydride and 6% 2,6-lutidine in DMF, 5 min	3% acetic anhydride and 3% pyridine in DMF. 2x (2 min and 5 min)
Washing	5x with 80 µl DMF	20 ml DMF (2 min and 5 min) 20 ml ethanol (2 min and 5 min) 20 ml DMF (2 min and 5 min)
Deprotection	30 µl 20% piperidine in DMF (v/v), 2x (1 and 5 min) with one DMF washing step in-between	20 ml 20% (v/v) piperidine in DMF 2x (2 min and 5 min)
Washing	5x with 80 µl DMF	20 ml DMF (2 min and 5 min) 20 ml ethanol (2 min and 5 min) 20 ml DMF (2 min and 5 min)
BPB staining	----------	600 µl BPB solution (10 mg/ml in DMF) in 20 ml DMF, 7 min incubation, washing with ethanol until washing solution is colourless

Generally, production of PNA-arrays can be performed in two different ways. Either the PNAs are synthesised *in situ* or pre-fabricated PNA-molecules are spotted to an appropriate solid support (Figure 1). Here, protocols for both processes are presented. Since for microarray production only small amounts of PNA but very many different sequences are needed, a fully automated and parallel, thereby low-scale and low-cost PNA-synthesis in micro-well plates was established for the latter approach. The amount of synthesised PNA can be controlled by the amount of resin applied to each micro-well, making the process scalable. Since standard HPLC purification is time-consuming and cost-intensive, protocols were set up for a process-inherent on-chip purification process.

Protocols and Results

All protocols presented here are based on standard Fmoc peptide chemistry. Therefore, all kind of Fmoc-protected linkers as well as amino acids and labelling reagents, which are commercially available for peptide synthesis, can easily be introduced into a growing PNA-oligomer chain. PNA synthesis is based on consecutive cycles of coupling a monomer to the growing chain, capping of unreacted, not elongated amino groups and cleavage of the Fmoc protection group (deprotection), with several washing steps in between. For both processes – *in situ* synthesis on the array surface and synthesis on a resin in micro-well plates – each synthesis step was refined for optimal yields. The resulting parameters are summarised in Table 1.

Chemicals

Fmoc-protected PNA-monomers as well as Fmoc-AEEA-OH linker, 1-hydroxyazabenzotriazole (HOAt) and O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) were obtained from Perseptive Biosystems Inc. (Framingham, USA). Fmoc-protected amino acids and acid cleavable Fmoc rink-linker were purchased from Novabiochem-Calbiochem (Läufelfingen, Switzerland), and 1-methyl-2-pyrrolidone (NMP) from Sigma-Aldrich (Munich, Germany). The rink resin LS used for the synthesis in micro-well plates was from Advanced ChemTech (Louisville, USA). Unless stated otherwise, all other chemicals and solvents were purchased from Fluka (Steinheim, Germany), Sigma-Aldrich or SDS (Peypin, France) and used without further purification.

Microarray Production by Spotting Pre-Fabricated PNA-Molecules

Parallel PNA-Synthesis in Micro-Well Plates

As shown in Figure 1, low-scale, parallel PNA-synthesis took place in microtitre plates with integrated frits by means of an AutoSpot

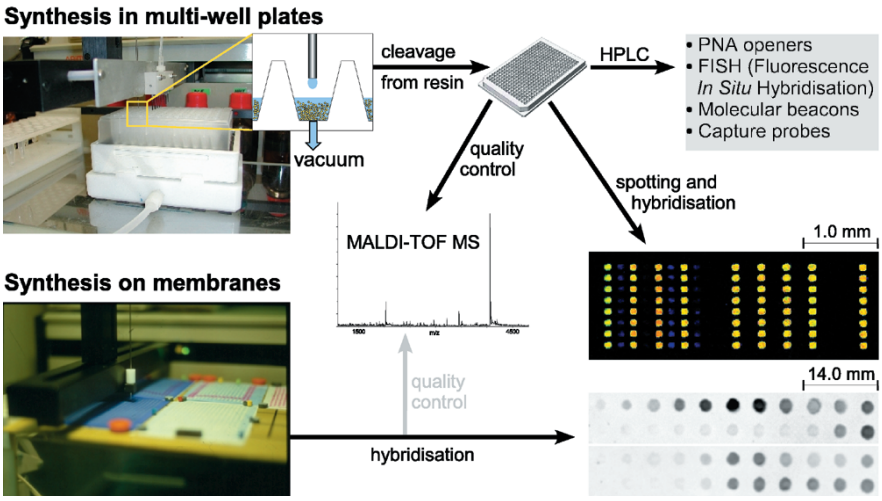

Figure 1. Schematic representation of the two procedures established for the creation of PNA-arrays. PNA-synthesis occurs either in resin-filled micro-well plates or by *in situ* synthesis directly on the array surface. In the former process, the finished PNA-products are cleaved from the resin and transferred into a new plate prior to spotting them on glass or silicon slides. Because of selective binding, only full-length molecules get attached to the microarray slide.

pipetting robot (INTAVIS Bioanalytical Instruments AG, Cologne, Germany). The robot was connected to a vacuum device that extracted the reagents from the wells during the synthesis cycles. Fmoc-protected rink resin LS (100-200 mesh, substitution of 0.2 mmol/g) was swelled in dimethylformamide (DMF) at a concentration of 20 mg/ml for 1 h. A volume of 100 µl (2 mg resin) of this well-mixed suspension was placed in each well of the microtitre plate. Synthesis took place overnight in a fully automatic manner, each cycle starting with the Fmoc deprotection of the rink resin followed by washing the resin thoroughly with DMF. Subsequently, the respective monomer was coupled. Detailed reaction conditions for the entire cycle are given in Table 1. For each well, the desired PNA-sequence was fed into the AutoSpot software that then calculated, which total volume of monomer solution and activating reagent had to be mixed. Only the amount of activated PNA-solution actually required for each cycle was made up. Coupling experiments had shown a gradual loss of coupling activity after 1 hour of activation (3). Reagents were

thoroughly mixed by aspirating and dispensing. After pre-activation, the dispenser needle pipetted 8 µl of activated monomer solution into each well. Once one monomer was placed in all appropriate wells, the other three were dispensed the same way. In order to compensate for the lack of any stirring or other mixing process within the wells, two subsequent coupling steps were performed for improved coupling yields. Washing with DMF was done with the multichannel dispenser arm, while capping and deprotection reagents were delivered sequentially by the dispenser needle.

After finishing the last synthesis cycle with the removal of the Fmoc protection group, the resin in each well was washed five times with 80 µl DMF followed by three washing steps with 80 µl 1,2-dichloroethane (DCE). The PNAs were fully deprotected and cleaved from the resin by incubation with 70 µl 80% trifluoroacetic acid (TFA) with 5% triisopropylsilane in dichloroethane for 1 h. If the molecules were to be used in applications other than microarray production, they were eluted from the resin with another 140 µl 80% TFA mixture and transferred into a new micro-well plate. In order to get rid of the Bhoc side chain protection groups, the PNA was precipitated by addition of 1 ml ice-cold diethyl ether. After complete evaporation of any ether remains, each product was dissolved in 100 µl water and stored at 4°C. Standard synthesis on 2 mg resin yielded about 100 nmol of PNA product, determined by adsorption measurements at 260 nm. This overall yield, however, could be adjusted by varying the amount of resin used during synthesis. For a fast and economic production of microarrays, the ether precipitation was avoided. The PNAs were eluted from the resin into another micro-well plate with 200 µl water instead of the TFA mixture, lyophilised and dissolved in 100 µl water. The eventual microarray performance was not affected by this simplification.

For the introduction of modifications like linker molecules, biotin, cysteine or other amino acids, the respective compound was applied in the same concentration as described above for the Fmoc-protected monomers. Since the robotic system can also be used for peptide synthesis, the number of monomer solutions, which will be mixed with activation reagents and dispensed automatically, is not limited to

four. Thus, even many different modifications could be introduced in a growing PNA-chain.

Quality Control

Quality control was performed mainly by MALDI-TOF mass spectrometry (MALDI-TOF MS). For this purpose, 0.5 µl of diluted PNA and 0.5 µl matrix (0.7 M 3-hydroxypicolinic acid, 70 mM ammonium citrate in 50% aqueous acetonitrile) were mixed directly on the target. The mixture was allowed to dry at ambient temperature and then introduced into the mass spectrometer for analysis. MALDI-TOF mass spectra were recorded in positive ion reflector mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik GmbH, Bremen, Germany) equipped with a 337 nm nitrogen laser. The whole PNA synthesis process was optimised with regard to reaction times, washing steps and the amount of reagents and resin by analysing the crude products. With the final protocol presented here, PNAs of up to 20 nucleotides in length were synthesised with good overall performance. Figure 2 shows typical mass spectra for two 13-mers, one modified with a biotin at the amino-terminus, and one 20-mer.

Production of PNA Microarrays

Even though PNA-synthesis usually worked well, truncated molecules resulted from each reaction nevertheless. Especially PNA-molecules containing many successive purines were difficult to synthesise. For normal purposes, PNAs are purified by reverse phase HPLC. For the production of PNA-microarrays, however, this would be too cost and labour intensive. However, a rapid and simple purification of the crude PNA-products could be achieved by a selective covalent binding of the terminal amino function of the full-length products to an appropriately modified surface. Here, aldehyde and succinimydyl ester activated surfaces were used. Both react selectively with primary amino groups. As all truncated sequences were capped and therefore acetylated, they could not bind to the solid support and were removed

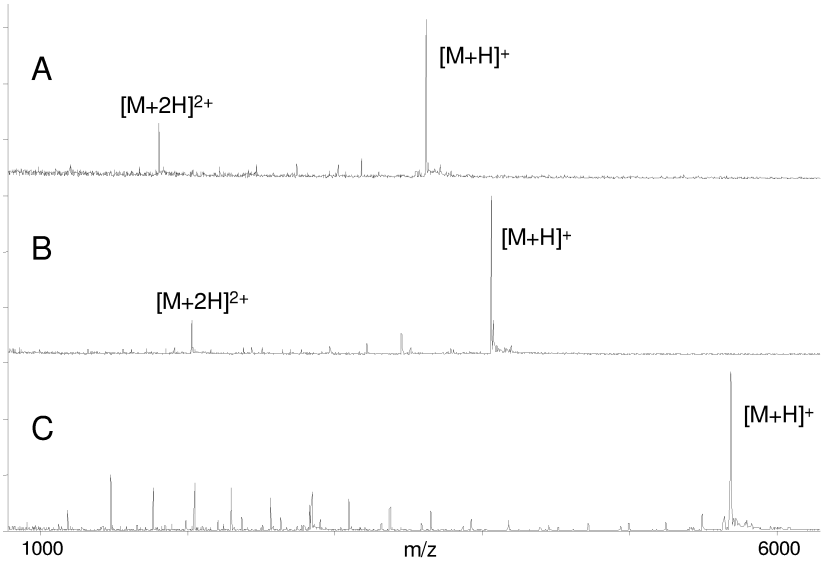

Figure 2. Quality assessment. Products of PNA-syntheses in micro-well plates were analysed by MALDI-TOF MS. From top to bottom, spectra of the following crude PNA-products are shown: A: Li-AAAACCGCTACTC, B: Biotin-Li-AGCTTACGGATCA-Li, C: Li-Li-CATACAAATTGCGGAATTTA (Li = AEEA-OH linker).

during washing. Thereby, spotting and purification took place concomitantly.

For the production of high-density microarrays, the crude PNA-products were spotted onto succinimidyl ester activated aminosilane surfaces (glass or silicon slides), with an SDDC-2 DNA Micro-Arrayer from Engineering Services Inc. (Toronto, Canada) and SMP3 pins (TeleChem International Inc., Sunnyvale, USA). Centre-to-centre spacing of the spots was 170 μm. Generally, spotting buffers with high salt content are used to avoid quick evaporation of solvent during the spotting process and the coupling reaction. However, a few PNA-sequences showed solubility problems in such buffers, which could not even be circumvented by introducing one or two terminal lysine residues or other modifications, which enhance solubility. Good results, however, were obtained with an aqueous 1 M betaine solution, the pH adjusted to 7.5 with NaOH. In this spotting solution, we observed

sufficient solubility of all sequences as well as selective and efficient covalent binding of PNA-oligomers to the microarray surface. In order to achieve uniform loading, a PNA-concentration of at least 160 μM was required. At this concentration, the activated surface of the succinimidyl ester slides exhibited saturation of its loading capacity.

Spotted slides were incubated overnight and then deactivated in a solution of 50 mM succinic anhydride and 150 mM 1-methylimidazole (NMI) in dichloroethane for 2 h. For the removal of all by-products – in form of acetylated sequences – and not covalently bound full-length PNA-molecules the following washing scheme was applied. The slides were washed twice with dichloroethane, rinsed then twice with buffer (5 mM sodium phosphate, 0,1% SDS) that had been heated to 90°C, and incubated in water of the same temperature for 10 min. Subsequently, the slides were rinsed with 1 M NaCl in aqueous 0,1% TFA and with water. After drying with nitrogen, they were stored at 4°C.

Quality Control of PNA-Microarrays

Two processes were used to monitor the efficiency of the on-chip purification by selective binding. First, Cy5-labelled, complementary oligonucleotides were hybridised to the arrays. Crude PNA-products as well as PNAs with a quantitatively acetylated amino function were spotted. Only spots of the former resulted in good hybridisation signals. Second, PNA-product with an artificially high amount of truncated, thus acetylated sequences was bound to a 5x5 mm succinimydyl ester activated silicone slide. The slide was modified with an acid cleavable rink linker, which was attached directly to the surface at one end and had a mass tag added to the other (Figure 3C). Binding of the crude PNA, deactivation of the surface and washing steps were carried out as described above. Covalently bound products were then cleaved off the support by adding 15 μl of 95% TFA, 5% triisopropylsilane (v/v) directly to the surface (3 times within 1 h). After evaporation of excessive TFA, 1 μl matrix was added and the chip was transferred directly into a mass spectrometer for analysis. By this process, covalently bound PNA could easily be detected by the mass shift caused

by the addition of the mass tag, which remained attached to the PNA upon cleavage of the rink linker. Any by-product of PNA-synthesis, such as the acetylated sequences, should also exhibit this mass shift, if covalently bound. Any molecule, which may have bound unspecifically to the surface, however, such as truncated sequences as well as excessive, unbound full-length product, for example, could be identified by their unchanged masses. MALDI-TOF spectra of crude PNA products and the corresponding analysis after attachment to the

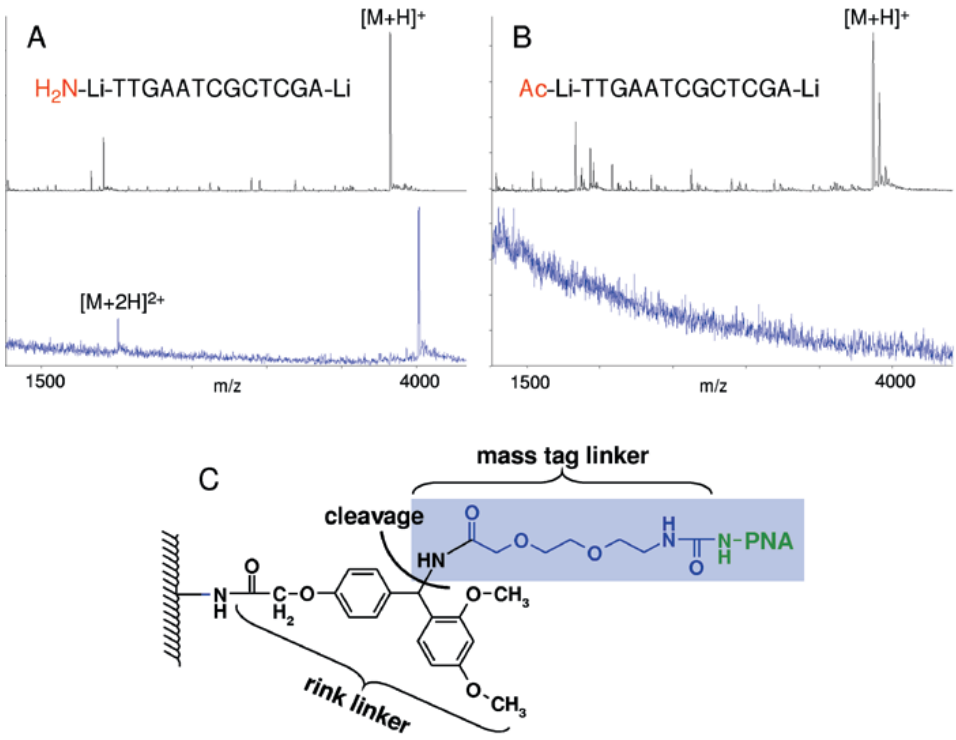

Figure 3. On-chip PNA purification. (A) Mass spectra are shown of a crude 13-mer, (top panel; $[M+H]^+ = 3827$ Da) and the same oligomer upon binding to and subsequent release from a microarray surface (bottom panel; $[M+H]^+ = 4015$ Da). The mass shift was caused by the cleavable linker, with which the chip was coated, and reveals covalent binding. Shorter derivatives were efficiently washed away. (B) The spectra show the results of an experiment identical to the above but performed with a quantitatively acetylated 13-mer (top panel $[M+H]^+ = 3869$ Da). The absence of signal in the lower spectrum demonstrates the lack of any covalent or unspecific binding to the microarray surface. (C) The structure of the used linker-system is shown.

slide surface and subsequent cleavage confirmed the selective binding via the terminal amino group (Figure 3A). As covalent binding of acetylated by-products via their exocyclic amino functions of the bases may happen but fall below the detection limit of MALDI-TOF MS, the experiment was also carried out with crude, fully acetylated PNA-product. Neither any covalently attached acetylated PNA nor signal of unspecifically bound PNA could be detected (Figure 3B). The hybridisation experiments and MALDI-TOF analyses indicated, however, that extensive washing – as described above – was essential for a good purification effect. Less washing resulted in additional signals due to unspecific binding to the surface. As a matter of course, the purification scheme described can also be performed with other selective coupling chemistries. Efficient purification of crude PNA-products has also been reported for molecules that carry a biotin or cysteine modification at their amino-terminus (Brandt *et al.*, unpublished).

Production of PNA-Arrays by *in situ* Synthesis

Synthesis on Membranes (Macroarrays)

In situ synthesis of PNA oligomer arrays was carried out analogous to the SPOT method developed by Ronald Frank for the parallel synthesis of spatially addressable peptide libraries (4). Basically the same robotic system was used as described above. Rather than resin in microtitre plate wells, however, membranes acted as solid support that had been mounted to a flat stainless steel plate (1). Either cellulose (WHATMAN 540) or polypropylene membranes with a Jeffamin 500 (O,O'-Bis-(2-aminopropyl)-polyethylenglycol 500) linker were used. In the case of cellulose, the starting monomer was attached to the surface via a base-cleavable ester bond. On polypropylene, the monomer was coupled directly via a stable peptide bond to the terminal amino group of the linker. Optionally, an acid-labile Fmoc-rink linker could be introduced as the first monomer. This was particularly useful for the purpose of quality control. Detailed synthesis conditions are shown in Table 1. The dispensing time required was dependent on the number of spots and their position. In addition to the duration of the mere spotting, 15

min were usually added to achieve completion of the condensation also for the reaction spotted last. For a grid of 1,720 spots, a spotting cycle took altogether about 55 minutes.

Contrary to the fully automatic synthesis in micro-well plates, the capping, washing and deprotection steps had to be performed manually. The membrane was transferred into flat polypropylene containers and 20 ml of the respective reagent were added under moderate shaking. If the acid cleavable rink linker was used, addition of the base pyridine to the capping reaction was essential, since otherwise the growing PNA chain could be cleaved partially from the solid support. After deprotection and washing, the membrane was mounted to the synthesiser again for the next cycle.

After synthesis, the Bhoc side chain protection groups were removed by treatment with a mixture of 25% TFA, 5% triisopropylsilane and 70% dichloroethane for 2 x 15 minutes. As the rink linker proved to be stable under these conditions, PNA-arrays on rink-modified polypropylene membranes were treated the same way. PNAs on polypropylene without rink linker can also be deprotected with 95% TFA, 5% triisopropylsilane for 10 minutes. Fully deprotected PNA-membranes were washed with dichloroethane and ethanol. After drying, they could be used directly for hybridisation experiments. Otherwise, they were stored in a sealed plastic bag at -20°C.

Quality Control

Optionally, staining the membranes with bromophenol blue after each Fmoc deprotection (Table 1) could check the effectiveness of the synthesis. Stained membranes show a blue signal at each PNA spot, where there are free (basic) amino functions. The signal intensity is indicative for the number of molecules that can be extended during the next condensation reaction. The blue colour disappears, when the next monomer is coupled to the free amino group.

Quality control of synthesis products could be performed, provided that rink linker polypropylene membranes were used. Spots were cut

out and the oligomers released by a treatment with 95% TFA, 5% triisopropylsilane for 40 min. After lyophilisation, the crude product was dissolved in water (10 µl per spot) and analysed by MALDI-TOF MS and reverse-phase HPLC. For the latter, a capillary HPLC-system equipped with a reverse-phase column (PepMap, C18, 3 µm, 0,3 x 150 mm), a 140B solvents delivery system (Applied Biosystem, Foster City, USA), accurate splitter (LC-Packings, Amsterdam, The Netherlands), UV absorbance detector 759A, U-Z capillary flow cell (LC-Packings) and a fraction collector were used. Detection wavelength was 260 nm. Separation was performed using a flow rate of 4 µl/min and a linear gradient (Eluent A: 0.1% TFA in water; eluent B: 0.1% TFA in acetonitrile. Elution started with 4% B and 96% A for 10 min, proceeded with 4-15% B in 40 min, 15-20% B in 5 min, 20-100% B in 5 min., additional 5 min at 100% B and then from 100-0% B in another 5 min). Usually fractions of about 4 µl were collected. HPLC chromatograms of the crude PNAs showed a lot of signal peaks deriving from small aromatic compounds, which got stuck to the membrane. Therefore, fractions that gave a signal in the HPLC chromatogram were analysed by MALDI-TOF MS. This yielded not only an assignment of the signals but also demonstrated that the gradient used was suitable for separating the full-length products from the shorter derivatives. The gradient listed above worked well for HPLC analyses of PNAs of up to 14 nucleotides in length.

Reverse-phase HPLC was also used to quantify the amount of full-length product. Samples were run in parallel to a standard of two pure PNA-oligomers of defined lengths and sequences. Several HPLC runs were performed with different concentrations of this standard mixture. Quantification of the peak areas of the standard PNAs, also considering the different extinction coefficients caused by the base composition, resulted in a calibration curve, based on which the concentration of a sample PNA was calculated. Typical yields were in the range of 40 pmol/spot.

Quality control by MALDI-TOF analysis demonstrated that generally synthesis worked well for PNAs of up to 14-mers. For longer or purine-rich sequences, the percentage of shorter by-products increased significantly. Reproducibility was quite good (Figure 4), considering

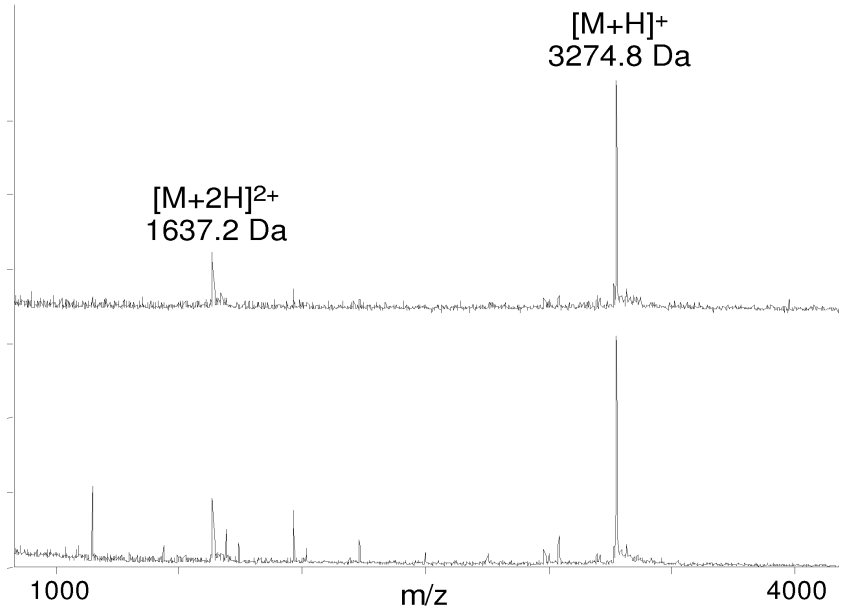

Figure 4. Reproducibility of *in situ* PNA-synthesis. A 12-mer PNA (AGCTTACGGATC) was synthesised in different membrane spots. MALDI-TOF MS spectra of the released, crude synthesis products are shown.

the fact that the membranes were removed from the robotic device during each cycle and had to be realigned for application of the next monomer.

Synthesis on Silicon or Glass Slides

Production of microarrays can also be achieved by *in situ* synthesis. Since the robotic system described above was incapable of delivering volumes smaller than 0.2 µl, pin-based contact printers or piezo-driven spotters were used to deliver the activated monomer. Reactions took place in organic solvents, which tend to spread on the surface. This resulted in spots bigger than those obtained with aqueous solutions. Whereas for the contact printing of aqueous PNA-solutions by split pins the centre-to-centre spot distance was 170 µm, a full millimetre was necessary with organic solvents. With piezo spotters that delivered

a well-defined volume of 330 pl per droplet, spot distances of 400 μm were possible. *In situ* synthesis on aminosilane-modified silicon or glass slides was usually carried out with the SDDC-2 DNA Micro-Arrayer (Engineering Services Inc., Toronto, Canada) and SMP3 pins (TeleChem International Inc., Sunnyvale, USA). Protocols for coupling chemistry, cycle procedures and cleavage of the Bhoc groups were basically identical to *in situ* synthesis on membranes but for the fact that much smaller volumes were needed. Only about 1 nl of activated monomer was applied per spot, for example. Coupling was performed only once and the coupling time was about 10 min. Also, bromophenol blue staining made no sense, since the concentration of free amino functions was too low to be visualised.

Quality Control

Quality was assessed by hybridising Cy5-labelled oligonucleotides. Good hybridisation signals could be obtained. MALDI-TOF analysis of the product proved to be difficult, since the amount of PNA per spot was close to the detection limit. Instead, spotting of larger volumes was done manually on rink handle modified silicone wafers. Cleavage revealed molecules that were very similar in quality to those obtained on membranes. However, there could be a difference between the products from this control experiment and the actual micro-spots. The small droplets may be more susceptible to moisture, which in turn may result in a higher percentage of aborted synthesis reactions.

Application in Hybridisation Assay

One possible application for PNA-arrays is the detection of point mutations for diagnostic purposes. For the lack of phosphates in PNA, such analysis could be performed without sample amplification and labelling. Genomic DNA could be directly hybridised to PNA-arrays. Binding would be scored by detection of the phosphates of the bound nucleic acids by secondary mass spectrometry (2). Since this process could be highly sensitive, prior sample amplification would be obsolete. Still, any analysis could work only, if hybridisation would be selective.

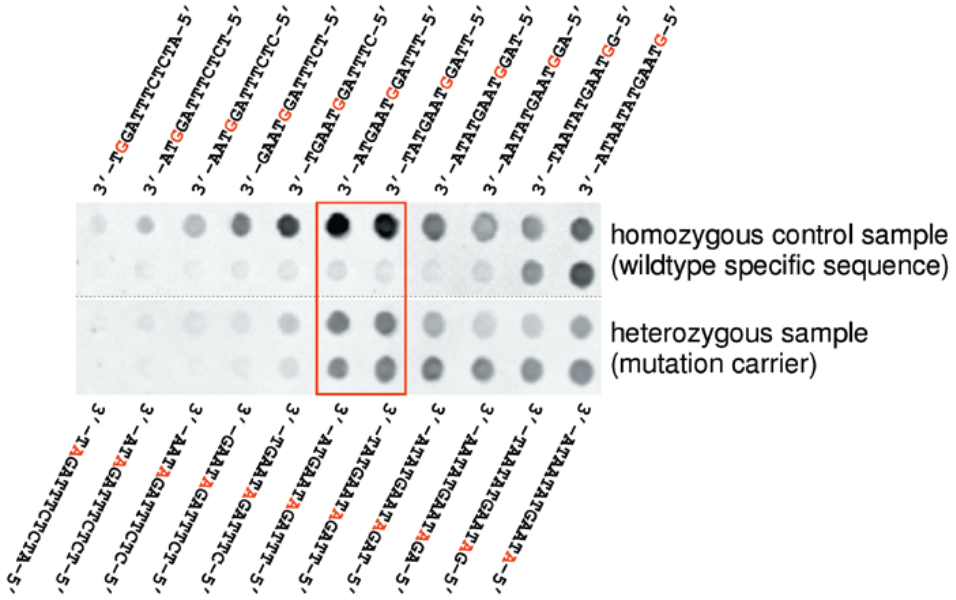

Figure 5. Discrimination of wildtype and heterozygous polymorphic sequence. Two rows of 12-mer PNAs were synthesised that represent a polymorphic region of the *BRCA1*-gene. The upper row of 12-mer PNAs – each shifted by one base – represents the wildtype sequence, the lower row the mutated sequence. The relevant DNA-region of a wildtype control and a heterozygous carrier of the mutation were PCR-amplified and hybridised to the array. The sequences shown next to the spots show the DNA-sequences that are complementary to the respective PNA-molecules; the polymorphic base is labelled in red. The red frame highlights the best performing oligomers.

We pursued several studies on PNA-microarrays, one aiming at the establishment of a diagnostic microarray for breast cancer genotyping. As part of this, a known point mutation (G to A) in the breast cancer susceptibility gene *BRCA1*, for example, was investigated for its occurrence in patient-DNA. In order to obtain probes, which exhibit a good discrimination between wildtype and heterozygous mutant sequence, the length of the PNA-molecules as well as the position of the point mutation within the PNA-oligomer were optimised. Figure 5 presents the results of an experiment for the latter purpose. Eleven 12-mers were synthesised *in situ* that covered the polymorphic site but were offset in position by one base each. Both the wildtype sequence and the corresponding mutant sequence were hybridised as

described in detail elsewhere (5). The membrane was hybridised with PCR-amplified wildtype samples, followed by a sample of a mutation carrier. Clear discrimination occurred on oligomers, which contained the polymorphism at a central location. Only these sequences were selected for the diagnostic microarray.

Discussion

PNA-arrays have come a long way from their first appearance several years ago (1). Considering the fact that PNA-monomers and thus also oligomers are rather expensive, low-cost production of PNA is required for an investigation of PNA/DNA or PNA/RNA interaction on microarrays. Both the PNA-synthesis in micro-well plates followed by spotting and the *in situ* synthesis permit array production at a reasonable cost. Either array format proved to be robust and stable and performed well in hybridisation experiments. Both approaches are flexible with regard to array design. Membranes, however, have several inherent disadvantages that are detrimental to their routine application. They lack mechanical stability, for example, which makes them difficult to handle. In addition, they are rather inflexible with respect to the mode of labelling and detection. Also, only one copy of an array is synthesised per run and the current synthesis protocol requires manual interference. In contrast, parallel synthesis in multi-well plates is a fully automated process. Beside the production of PNA-oligomers for microarrays, the resulting molecules can be used directly for other purposes, such as fluorescence *in situ* hybridisation experiments (6), applications as capture probes (7), molecular beacons (8) or PNA-openers (9, 10). The overall PNA yield can be adjusted to the actual need by adapting the amount of resin. With regard to microarray production, thousands of array copies can be produced from a single synthesis. In addition, only full-length molecules get attached to the array. *In situ* synthesis will only become competitive again, once appropriately adapted hardware for the production of high-density microarrays will exist. On glass or silicon slides, only few nanolitres of activated monomer are consumed per oligomer. However, a very high accuracy of the spotting device will be required, since the monomers will have to be delivered to exactly the same slide

coordinates many times over. Otherwise, an increasing percentage of truncated sequences will be created, mainly in the border area of each spot. The stepwise synthesis yield will not be limiting for many assays. Due to the high stability of PNA/DNA duplexes, only short molecules are required anyway. Besides, for applications like transcriptional profiling, for example, truncated molecules would not affect the analysis, as long as they hybridise to the same target sequence as the full-length product.

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