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Patterning of Peptide Nucleic Acids Using Reactive Microcontact Printing†

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PNAs (peptide nucleic acids) have been immobilized onto surfaces in a fast, accurate way by employing reactive microcontact printing. Surfaces have been first modified with aldehyde groups to react with the amino end of the synthesized PNAs. When patterning fluorescein-labeled PNAs by reactive microcontact printing using oxygen-oxidized polydimethylsiloxane stamps, homogeneous arrays were fabricated and characterized using optical methods. PNA-patterned surfaces were hybridized with complementary and mismatched dye-labeled oligonucleotides to test their ability to recognize DNA sequences. The stability and selectivity of the PNA-DNA duplexes on surfaces have been verified by fluorescence microscopy, and the melting curves have been recorded. Finally, the technique has been applied to the fabrication of chips by spotting a PNA microarray onto a flat PDMS stamp and reproducing the same features onto many slides. The chips were finally applied to single nucleotide polymorphism detection on oligonucleotides.

Introduction

Peptide nucleic acids (PNAs)[1] have attracted a great deal of attention owing to their versatile synthetic accessibility and functionalizability,[2] high sequence specificity to cDNA,[3] enhanced duplex stability when hybridized with cDNA as compared to the stability of DNA-DNA duplexes,[4] and increased resistance to chemical and enzymatic degradation. In particular, replacing DNA probes with synthetic PNAs has been shown to improve significantly the sensitivity of diagnostic and detection devices for DNA analysis.[5] Moreover, it has been demonstrated that it is possible to introduce modifications within the backbone that can introduce improved binding properties. Chiral PNAs are among the most promising modified PNAs and consist of the introduction of substituents to position 2 or 5 of the backbone based on the structure of amino acid side chains.[15,16] It has been observed that the introduction of positively charged side chains (such as lysine or arginine side chains) has a series of effects that cannot be narrowed down only to an increased temperature due to electrostatic interactions[16c] but also causes improvements in terms of solubility and self-aggregation. The configuration of the stereogenic centers introduced into the backbone modifications affects the PNA preferential helicity, finely tuning the binding properties of such modified PNA molecules. The presence of a stereogenic center of the correct configuration (2D, 5L or 2D, 5L) has been demonstrated to introduce a preorganization of the system that causes an increase in the duplex stability as well as an improved selectivity.[15,16]

Although PNAs have been successfully applied in various detection schemes on surfaces,[6] methods to pattern PNA by microcontact printing (μCP), which would be advantageous for diagnostic bioapplications, have not been reported. μCP is a biocompatible method for patterning biomolecules directly onto surfaces[7] and represents an elegant route to attaching PNA to surfaces locally. Recently, it has been shown that μCP can also induce chemical reactions during printing.[8] An important feature of μCP is the short contact time necessary

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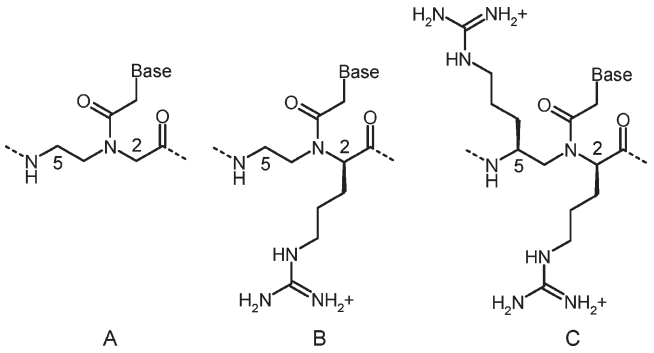

Figure 1. Structure of achiral (A), 2D-arginine (B, 2D-Arg), and 2D,5L-arginine (C, 2D,5L-Arg) PNA monomers.

to form a dense monolayer of ink on the substrate. Contact times are typically around minutes, but μCP has also been performed with millisecond contact of stamp and substrate. The products of this technique are highly defined and very reproducible[8] patterns. When a reaction is involved between molecules and a substrate, the time necessary is typically much shorter compared to the time necessary for the comparable reaction from solution. Moreover, when an array of various inks is robot-spotted onto a stamp, μCP can be used to generate multiple copies of this array on substrates.[9] PNAs have been immobilized using Huisgen 1,3-cycloaddition between azide surfaces and acetylene-carrying PNA,[10] thiolated PNAs on gold or maleimide surfaces,[11] amino-capped PNAs on isothiocyanate,[12] succinimidyl[13] or aldehyde surfaces,[14] and on surfaces partially prefunctionalized with cDNA.[6b] Here, we show the successful repetitive printing of chiral arginine-modified PNAs (Figure 1) by applying imine chemistry to bind PNA covalently to aldehyde-functionalized surfaces through its amino end. The labile imine bond is reduced to a stable secondary amine bond forming a robust connection between the PNA strand and the solid support. Subsequently, surface-bound PNA recruits complementary and fluorescently labeled DNA to the surface, which is analyzed with fluorescence microscopy.

Results and Discussion

PNA Patterning. To immobilize PNA on glass or quartz slides, surfaces were functionalized with aldehydes[9] and PNAs, which were derivatized with an amino group attached via a short aminoethyl ethoxy linker (AEEA), and were printed as depicted in Scheme 1. The PNA sequences used here are shown in Table 1. **PNA-1** carries a fluorescent label to study the patterning on the surface using fluorescence microscopy. For this reason, a lysine unit carrying a fluorescein dye was introduced into the PNA chain. For patterning, a freshly oxidized PDMS stamp was inked with a 20 μM solution of **PNA-1** in CH_3CN for 5 min at room temperature. The stamp was dried with N_2 and brought into contact with the aldehyde-modified glass or silicon oxide slides for 15 min by applying slight pressure (Scheme 1). Subsequently, the imine linkage between the peptide nucleic acid strands and the substrate was reduced to the corresponding amine with sodium borohydride to prevent hydrolysis (Scheme 1).

After sonication for 2 min in a solution of 0.1 M sodium carbonate and 2% SDS (pH 9) to remove unbound molecules, the substrate was washed with a 1 mM solution of sodium carbonate. Imine reduction did not lead to a decrease in signal intensity for printed PNA. Figure 2A−D shows fluorescence microscopy images taken after printing PNA with stamps inked with solutions of PNA at different concentrations (1, 5, 10, and 20 μM) and subsequent imine reduction. A decrease in the intensity of the patterns can be observed upon decreasing the ink concentration from 10 to 1 μM PNA solutions (Figure 2B−D), and an ink concentration of 20 μM resulted in no further increase in intensity (Figure 2A,B). For all inking concentrations, the PNA patterns were homogeneous and had clear edge resolution. From these fluorescent images, it can be concluded that PNA is transferred from the stamp to the substrates, in contrast with previous reports on DNA printing where the slightly negatively charged surfaces of the stamps prevented significant transfer of DNA to the surface even though hydrophilic (oxidized) stamps were used.[9] To verify the presence of a PNA monolayer on the surface further, an unpatterned monolayer of PNA was formed on quartz slides by printing with flat stamps and UV−vis absorption spectra were recorded (Figure 3). The spectra of the printed slides show the typical peak at 260 nm originating from the absorbance of the nucleobases, whereas in nonfunctionalized slides this peak is absent.

To investigate whether printed **PNA-1** was accessible for hybridization, the patterned substrates were incubated with 1 μM solutions of **DNA-c1** (Table 1). Hybridization with the cDNA strand was carried out for 2 h at room temperature in saline sodium citrate (SSC) buffer containing 300 mM NaCl, 34 mM citrate (SSC), and 0.1% sodium dodecyl sulfate (SDS). After a brief wash with SSC buffer and a subsequent 10-fold diluted SSC buffer, both the PNA probe (green fluorescein dye) and the target DNA (red TAMRA dye) strands were clearly visible in the fluorescence microscope images shown in Figure 2E−L. The fluorescence intensity of the PNA after incubation was unchanged (Figure 2I−L), indicating that no PNA was removed from the surface upon hybridization. The fluorescence ratio of **PNA-1** and the target **DNA-c1** was the same for 20, 10, and 5 μM, whereas it increased in the case of 1 μM, indicating that the higher PNA densities do not disturb the hybridization process.

PNA Hybridization Properties. To examine the specificity of the hybridization, the influence of single base pair mismatches was tested by incubating surfaces with patterned PNA in a solution of DNA (Table 1). **PNA-2** (5 μM) was printed on glass slides and subsequently reduced and hybridized for 90 min with the complementary (**DNA-c2**, 5 μM) and singly mismatched oligonucleotide (**DNA-c1**, 5 μM) both labeled with TAMRA. After washing with an SSC solution, the slides were immersed in 1 mL of SSC buffer containing 0.1% SDS and oligonucleotides

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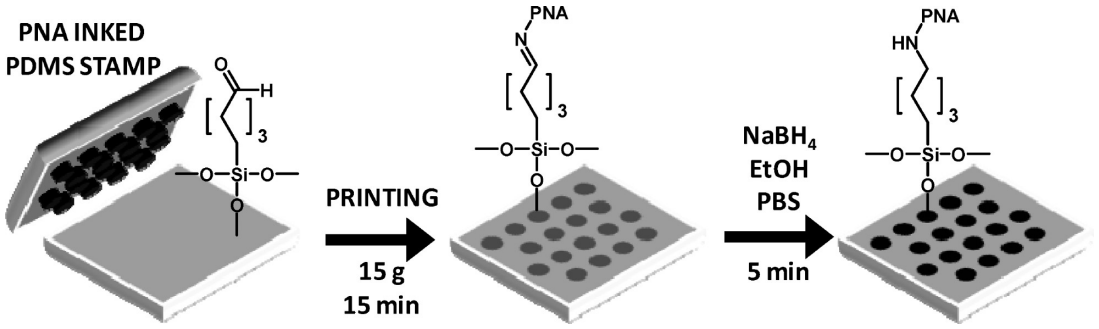
Scheme 1. Patterning of Amino-Capped PNAs onto Aldehyde-Functionalized Slides via Reactive Microcontact Printing[a]



[a] After reduction of the imine bonds, the PNA patterns were used for hybridization experiments.

Table 1. PNA Sequences and T_m of PNA/DNA Full Match/(Single) Mismatch Duplexes (5 μM) in 10 mM Phosphate Buffer (100 mM NaCl, 0.1 mM EDTA, pH 7)

PNA	sequence[a]	T_m (°C) PNA/DNA full match[b]	T_m (°C) PNA/DNA mismatch
1	$H(AEEA)_2TTACTCT_{(2D\text{-}Arg)}TCACC\text{-}Lys(fluorescein)\text{-}NH_2$	48 (with **c1**)	37 (with **c2**)
2	$H(AEEA)_2TTACTCA_{(2D\text{-}Arg)}TTCACC\text{-}NH_2$	55 (with **c2**)	35 (with **c1**)
3	$H(AEEA)_2GCCGCA_{(2D,5\ L\text{-}Arg)}CACGT\text{-}NH_2$	78 (with **c3**)	61 (with **c5**)
4	$H(AEEA)_2CAGGCA_{(2D,5\ L\text{-}Arg)}CTTCT\text{-}NH_2$	69 (with **c4**)	50 (with **c6**)
5	$H(AEEA)_2GCCGCG_{(2D,5\ L\text{-}Arg)}CACGT\text{-}NH_2$	85 (with **c5**)	69 (with **c3**)
6	$H(AEEA)_2CAGGCG_{(2D,5\ L\text{-}Arg)}CTTCT\text{-}NH_2$	75 (with **c6**)	60 (with **c4**)

[a] The chiral monomer corresponding to the SNP position is underlined in bold. [b] DNA sequences: DNA-**c1**: 5′-GGTGAAAGAGTAA-3′; -**c2**: 5′-GGTGAATGAGTAA-3′ -**c3**: 5′-ACGTGTGCGGC-3′; -**c4**: 5′-AGAAGTGCCTG-3′; -**c5**: 5′-ACGTGCGCGGC-3′; -**c6**: 5′-AGAAGCGCCTG-3′. AEEA: aminoethoxyethoxyacetyl.

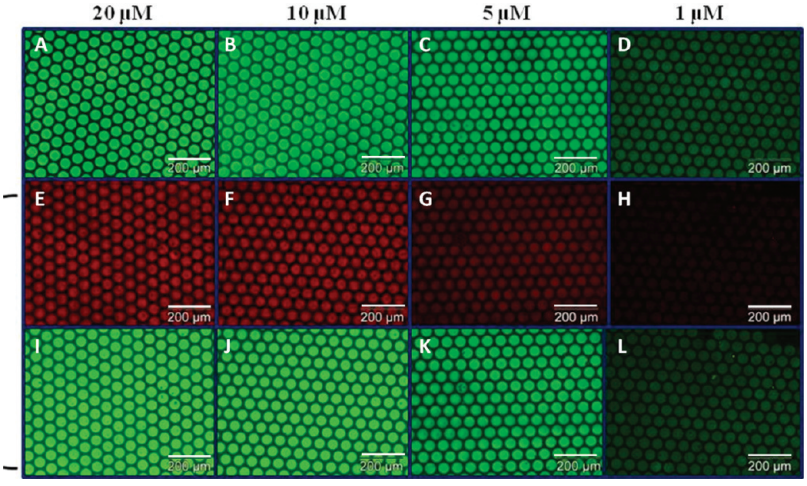


Figure 2. Fluorescence microscope images (scale bars 200 μm) of patterned fluorescein-labeled **PNA-1** (A−D), of TAMRA-labeled **DNA-c1** (20, 10, 5, and 1 μM) hybridized to patterned **PNA-1** (E−L), and of **PNA-1** after hybridization (I−L). Images E−H were recorded with λ_{exc} = 525 nm, λ_{em} = 550 nm, and images A−D and I−L were recorded using λ_{exc} = 475 nm, λ_{em} = 520 nm. Patterns were obtained by printing with freshly oxidized PDMS stamps that had been inked with 1, 5, 10, and 20 μM **PNA-1** solutions.

(10 nM) and imaged using a confocal fluorescence microscope at different temperatures between 20 and 40 °C while still immersed. The recorded images (Figure 4) show an intense, well-defined pattern at room temperature in both cases, but as expected, the fluorescence decreases upon increasing the temperature because of the melting of the duplexes and the release of labeled DNA into the buffer. The average fluorescence intensity was determined from three randomly chosen areas of the series of images both inside (PNA/DNA) and outside (background) the dots. The average background intensities were subtracted from the respective average fluorescence intensities. Plotting these values versus temperature resulted in a sigmoidal curve characteristic of nucleic acid duplex melting behavior. The melting temperature of the duplex with the singly mismatched DNA was determined to be 30 °C, whereas for the melting temperature of the duplex with the fully matched DNA 35 °C was found.

This observation shows that the linkage on the surface causes an important decrease in stability but conserves the ability to recognize mismatches, as in solution; however, the difference in melting temperatures was less pronounced when compared to solution data by 15 °C (Table 1). This behavior is currently subject to further detailed experiments.

To test the selectivity of printed PNA on the surface further, **PNA-3**, **PNA-5**, and **PNA-6** were used (Table 1). These PNA molecules have been previously used to study the recognition of two single nucleotide polymorphisms (SNPs) on gene APOE

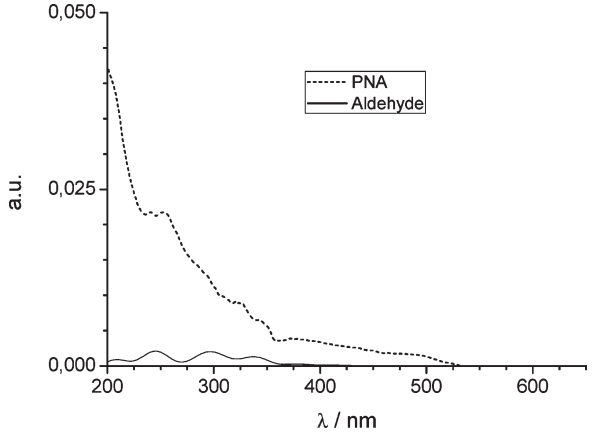


Figure 3. UV−vis absorption spectra of aldehyde-functionalized quartz slides before (−) and after (···) **PNA-1** functionalization and reduction.

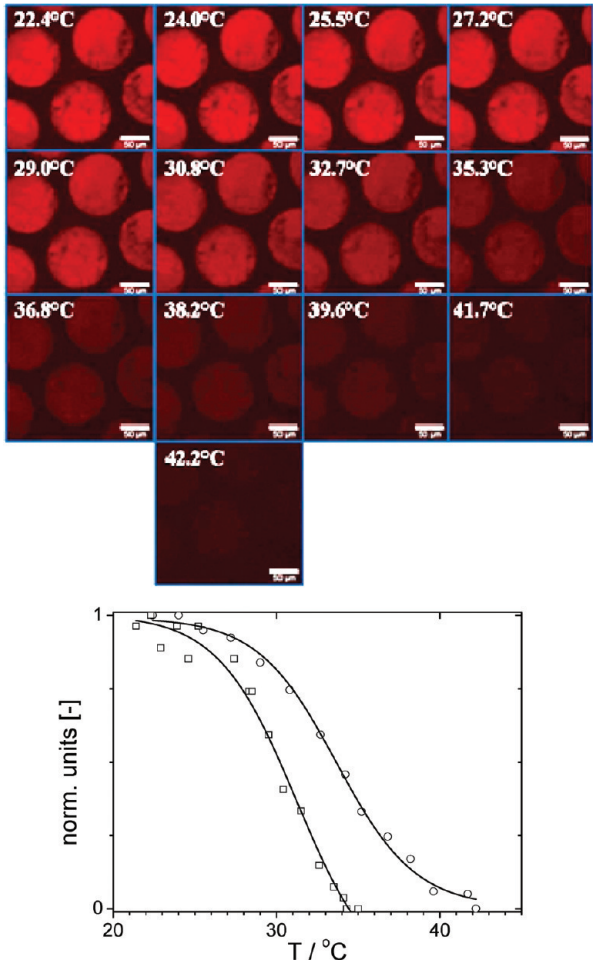


Figure 4. Confocal fluorescence microscope images (scale bars 50 μm) of TAMRA-labeled **DNA-c2** hybridized to **PNA-2** patterns as a function of temperature. The graph shows the melting behavior of **PNA-2** hybridized with fully matched (○, **DNA-c2**) and singly mismatched (□, **DNA-c1**) DNA.

related to the three isoforms (ε2, ε3, and ε4) of apolipoprotein E.[13] After these PNAs were printed on distinct areas on one glass slide, reduced, and hybridized with TAMRA-labeled **DNA-c3** and **DNA-c5**, the slides were analyzed using a fluorescence microscope. **DNA-c3** and **DNA-c5** are matched, singly mismatched, and noncomplementary strands for **PNA-3**, **PNA-5**, and **PNA-6** (Table 1). The hybridization was carried out at 50 °C for 4 h in SSC buffer containing 1% SDS and 1 μM DNA. Subsequently, the slide was washed with SSC buffer containing 0.1% SDS for 5 min at 50 °C and quickly rinsed with 10-fold-diluted SSC buffer at room temperature. The fluorescence microscopy images are shown in Figure 5. A distinct fluorescent pattern of significant intensity was observed in all cases of fully matched duplexes (Figure 5A,B,D,H), whereas in all cases of mismatched duplexes no distinct patterns could be observed (Figure 5C,E−G,I).

From these observations, we conclude that **PNA-3** and **PNA-5** selectively recognized a point mutation in **DNA-c3** and **DNA-c5**, respectively, but **PNA-6** did not show an affinity for any of these sequences, as was to be expected. When a mixture of **DNA-c3** and **DNA-c5** was used, both **PNA-3** and **PNA-5** showed comparable fluorescence intensities, whereas no fluorescent patterns were observed in the area where **PNA-6** was printed. These results are in line with our previous findings;[13] however, with our current fabrication method, the patterning is easier, homogeneous, and more versatile.

PNA Microarray Fabrication. To expand this method to multiple probe printing, we have used a commercial piezoelectric arrayer to fabricate arrays by the noncontact spotting of fluorescein-labeled **PNA-1** (10, 20, 50, and 100 μM) on flat, oxidized PDMS stamps with an average spot size of 200 μm. The PNA-spotted stamp was brought into contact with an aldehyde-derivatized glass slide for 15 min (15 g) and was subsequently used for another four prints without reinking with PNA. After reducing and washing the printed slides as described above, the slides were imaged using a fluorescence microscope (Figure 6). The microarray on the stamp was replicated successfully as a microarray on the aldehyde slide. The spot size is regular (200 μm in diameter), and the probe density is homogeneous. The first three prints exhibited almost equal intensities and a significant decrease in intensity was observed for the fourth print (Figure 6).

Although three prints with the same intensity may not seem to be a big step forward, there are several advantages for using the microcontact printing technique developed here over the commonly used direct spotting technique: (i) the printing time (15 min) needed for the transfer and covalent attachment of the PNA is a lot faster than the normal incubation time (overnight) needed for covalent PNA attachment when using direct spotting; (ii) the spots obtained by microcontact printing are more homogeneous, and defects commonly observed for direct spotting such as doughnut formation are avoided; (iii) PNA inking solutions for printing do not require the use of buffers or additives, and parameters such as temperature and humidity are not critical; and (iv) spotting needs to be done only once, and several prints can be obtained without reinking.

In a second experiment, we spotted different concentrations of **PNA-3**, **PNA-4**, and **PNA-5** (1, 5, 10, 20, 50, and 100 μM) onto a flat, oxidized PDMS stamp. After printing on an aldehyde slide and reducing, these arrays were hybridized at 55 °C for 3 h with TAMRA-labeled **DNA-c3** and **DNA-c5** (0.1 μM) in SSC buffer containing 0.1% SDS. The recorded fluorescence images in Figure 7 show the selective recognition of **PNA-3** toward **DNA-c3** and **PNA-5** toward **DNA-c5**, whereas in the case of **PNA-6** no affinity to either of these two DNA targets was observed, which is in agreement with the solution data (Table 1).

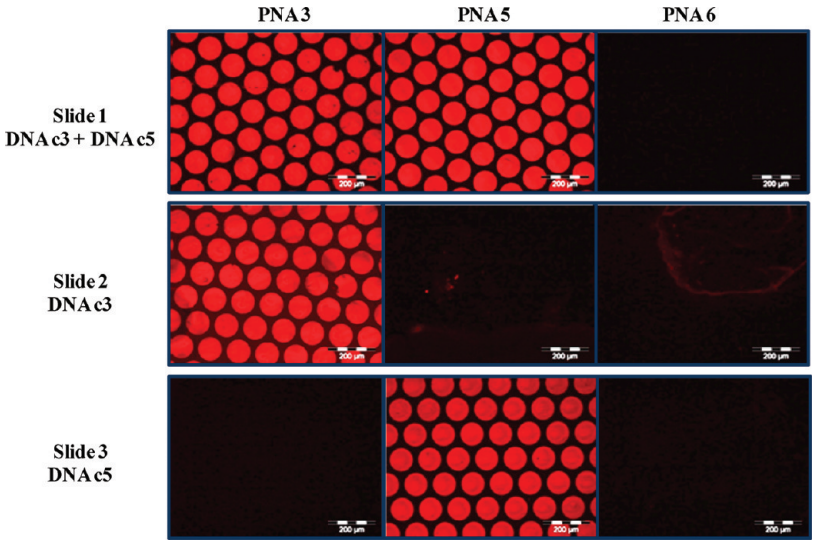


Figure 5. Fluorescence microscope images (scale bars 200 μm) of PNA slides (ink concentration 10 μM) hybridized with fully matched (**DNA-c3**) and singly mismatched (**DNA-c5**) fluorescently labeled DNA (concentration 1 μM).

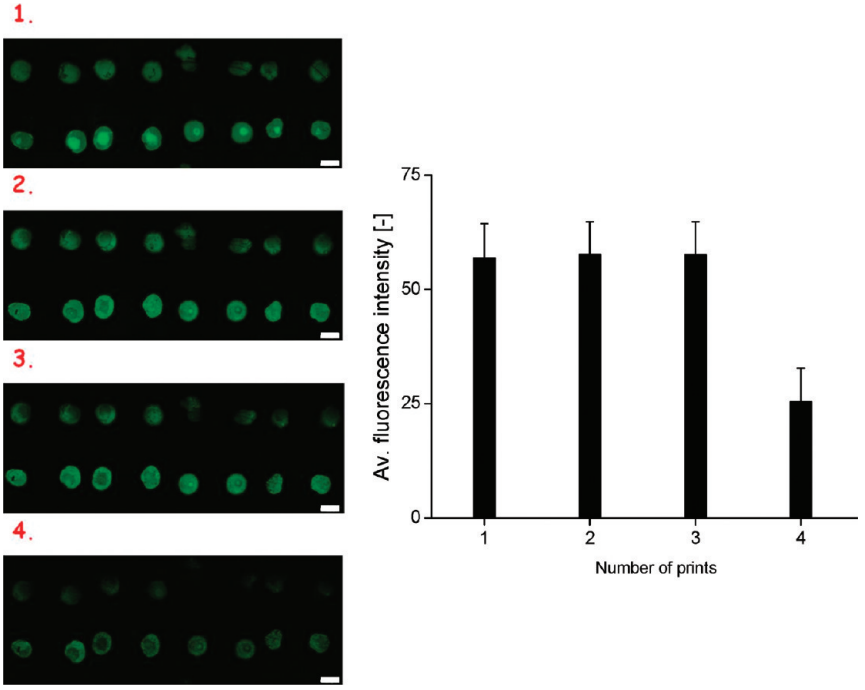


Figure 6. Fluorescence microscope images (scale bars 200 μm) of a microarray of flourescein-labeled PNA printed with a flat stamp onto which a PNA array had been spotted using a noncontact spotting arrayer.

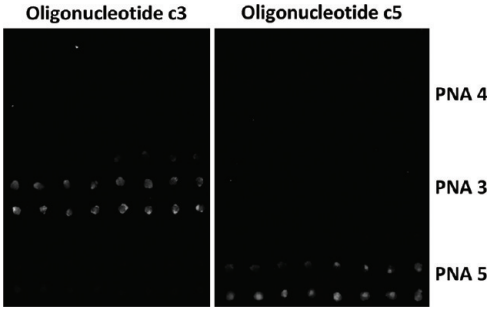


Figure 7. Fluorescence microscope images of TAMRA (Cy3 filter)-labeled **DNA-c3** (left) and **DNA-c5** (right) hybridized to a PNA microarray (**PNA-3**, **PNA-4**, and **PNA-5**). The PNA microarrays were printed using a flat stamp onto which a PNA array had been spotted using a noncontact spotting arrayer.

Conclusions

We have demonstrated that PNA patterns can be fabricated using reactive μCP. We extended this technique to print microarrays with spots of different PNA samples on glass slides in a single printing step. We did this by employing a noncontact robot arrayer first to spot the PNA microarray on a flat stamp and subsequently transfer printing it onto the slide. In this way, multiple replicas were produced without reloading the stamp. Arrays produced in this manner can be used for PNA/DNA hybridization experiments. We have demonstrated that our PNA microarrays can be used to distinguish between fully matched and singly mismatched complementary and mismatched DNA strands. We believe that our method of producing patterned surfaces displaying different PNA strands will be beneficial for the further development of various biosensor applications in high-throughput

formats. The method described here combines the possibility to spot several probes in a small area (typical for traditional array spotting techniques) with the possibility to have a short reaction time, more efficient reactions between the probes and the surface, and more reproducible feature definition between arrays and within the same array, as introduced by the microcontact printing method. This can make the PNA array fabrication step easier and cheaper and improve the quality of the devices thus fabricated, obtaining more effient diagnostic methods.

Experimental Section

Oligonucleotides were purchased from Sigma or MWG eurofins and used as received without further purification. All buffers and solutions were prepared using doubly distilled (18 MΩ cm) water. The following materials and chemicals were used as received: poly(dimethylsiloxane) (PDMS, Dow Corning), $NaBH_4$ (Aldrich), and trimethoxysilylalkylaldehyde (Fluorochem). All solvents were HPLC grade, and all other reagents were analytical grade. PNA *N*-Boc-protected standard monomers, *N*-[1*H*-1,2,3-(benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), *N*-[(dimethylamino)-1*H*-1,2,3-triazol[4,5-b]pyridine-1-ylmethylene]-*N*-methyl methanaminium hexafluorophosphate *N*-oxide (HATU), dichloromethane, *N*-methylpyrrolidone (NMP), and *N*,*N*-dimethylformamide (DMF) were purchased from Applied Biosystem (Foster City, CA). Carboxymethyl-*O*-benzyl-guanine was purchased from ASM (Hannover, Germany). Boc-Arg(Tos)-OH, Boc-D-Arg(Tos)-OH, and *m*-cresol were from Fluka (Buchs, Switzerland). (4-Methylbenhydryl)amine (MBHA) PS resin was from Novabiochem (Laufelfingen, Switzerland). Diisopropylethylamine (DIPEA), *N*,*O*-bistrimethylsilyl acetamide (BSA), trifluoromethane sulfonic acid (TFMSA), trifluoroacetic acid (TFA), 3-hydroxy-1,2,3-benzotriazin-4-(3H)-one (DhBtOH), and diisopropylcarbodiimide (DIC) were from Sigma-Aldrich (St. Louis, MO).

PNA Synthesis. The synthesis of chiral 2D-Arg-based PNAs was performed as described previously for similar PNAs[17] by using manual synthesis for the chiral residues and automated Boc-SPPS protocols on a ABI 433A synthesizer, following the procedures provided from the company, for the achiral residues. The chiral submonomers were introduced by using the HBTU/DIEA coupling protocol, the Fmoc group was deprotected by piperidine/NMP (20%), and carboxymethyl-*Z*-adenine[18] or carboxymethyl-*O*-benzyl-guanine (commercialy available) were linked to the submonomers on resin by using the DIC/DhBtOH coupling protocol because the PNA 1 Dde group was deprotected by hydrazine/DMF 2% and carboxyfluorescein was linked by the DIC/DhBtOH coupling protocol. Free PNAs were cleaved from the resin by using a 1:3 TFMSA/TFA mixture (10% thioanisole + 10% *m*-cresol) and precipitated by Et_2O. HPLC analysis for all PNAs was carried out by LC-MS by using an XTerra analytical C18 column (3 × 250 mm^2, 5 μm, flow 0.5 mL/min) and gradient elution from 100% H_2O (0.2% HCOOH, eluent A) to 60% H_2O and 40% CH_3CN (0.2% HCOOH, eluent B) in 30 min. The MS detector was set in positive ion mode with a capillary voltage of 3 kV, a cone voltage of 30 V, and full scan acquisition from 150 to 1500 *m*/*z*. HPLC purification for all PNAs was carried out on a semipreparative Jupiter (Phenomenex) C18 column (10 × 300 mm^2, 5 μm, flow 4 mL/min) with eluent A consisting of 100% H_2O (0.1% TFA) and eluent B consisting of 60% H_2O and 40% CH_3CN (0.1%T FA). Specific preparative elution conditions for every PNA are given below.

PNA-1. H-$(AEEA)_2$TTACTCT$_{(2D\text{-}Arg)}$TTCACC-Lys(fluorescein)-NH_2. The synthesis was performed on 25 mg of a preloaded Fmoc-Lys(Dde)-MBHA-PS resin (loading 0.2 mmol/g). Crude yield: 70%. The crude products were purified by semipreparative RP-HPLC via gradient elution: from 100% A (H_2O + TFA 0.1%) to 100% B (CH_3CN + 0.1% TFA) in 30 min. ESI-MS calcd *m*/*z*: 1075.1 (MH_4^{4+}), 860.6 (MH_5^{5+}), 717.3 (MH_6^{6+}), 615.0 (MH_7^{7+}); found *m*/*z*: 1074.2, 859.9, 716.7, 614.5.

PNA-2. H-$(AEEA)_2$TTACTCA$_{(2D\text{-}Arg)}$TTCACC-NH_2. Crude yield: 88%. The crude product was purified by RP-HPLC via gradient elution: from 100% A to 100%B in 30 min. ESI-MS calcd *m*/*z*: 956.0 (MH_4^{4+}), 764.9 (MH_5^{5+}), 637.6 (MH_6^{6+}), 546.7 (MH_7^{7+}); found *m*/*z*: 955.8, 764.8, 637.8, 546.7. The synthesis and characterization of chiral (2D,5L)-Arg submonomer chiral (2D,5L)-Arg-based PNAs (PNA-3 to PNA-6) were described in a previous paper.[13]

Substrate Functionalization. Clean microscope cover glass (Paul Marienfeld Gmbh & Co. KG, Germany) and quartz slides were activated in piranha solution for 30 min (concentrated H_2SO_4 and 33% aqueous H_2O_2 in a 3:1 ratio), rinsed with doubly distilled water, and functionalized in vacuo with trimethoxysilylalkylaldehyde overnight. (***Warning!*** *Piranha solution should be handled with caution. It has been reported to detonate unexpectedly*.) Substrates were rinsed with toluene to remove any excess silane and subsequently dried in N_2. We have carried out a control experiment where we immobilized a PNA probe (with amine functionality) onto a surface without aldehyde functionalities. In this case, there was no PNA discernible on the slides by means of fluorescence imaging.

Stamp Fabrication. Silicon wafer-based masters with etched structures were prepared by UV photolithography. The master's surface was fluorinated using fluorosilanes. PDMS stamps were fabricated by curing Sylgard 184 on the surface of the master at 60 °C overnight.

Microcontact Printing of PNA. PDMS stamps were first oxidized in a UV plasma reactor (Ultra-Violet Products, model PR-100) for 30 min at a distance of about 2 cm from the plasma source using a low-pressure Hg UV light (185 nm, 1.5 mW cm^{-2} and 254 nm, 15 mW cm^{-2}) as the light source. The stamps thus obtained were stored in Milli-Q water. The hydrophilic stamps were dried with N_2 and incubated with a drop of PNA solution in acetonitrile for 5 min at room temperature. The stamps were dried with N_2 and brought into contact with aldehyde-functionalized surfaces for 15 min at 15 g cm^{-2} of pressure. After being printed, the slides were immersed in a solution containing 100 mg of $NaBH_4$ in 40 mL of PBS with 10 mL of EtOH for 5 min. After being rinsed with doubly distilled water and sonicated in a 0.1 M carbonate buffer at pH 9 with 2% SDS for 2 min, the slides were rinsed with 1 mM carbonate buffer and dried with N_2.

Hybridization Experiments. Oligonucleotides were diluted to 1 μM in 2× SSC containing 0.1% SDS and applied to the surface of the modified slides using a hybridization chamber (Gene Frame, Thermo Scientific, U.K.). The temperature was tuned depending on the affinity of the probe for the complementary sequence. Then the samples were washed with SSC and 0.1% SDS at the hybridization temperature for 5 min and rinsed with 10-fold-diluted SSC buffer at room temperature for 1 min. After being washed, the glass slides were dried with nitrogen and analyzed on a fluorescence microscope (Olympus inverted research microscope IX71). The fluorescence ratios of **PNA-1** and the target **DNA-c1** belonging to Figure 2 are calculated from the following numerically averaged (corrected for background) fluorescence intensity values: PNA/DNA (170/95 (20 μM), 120/70 (10 μM), 105/55 (5 μM), and 50/17 (2 μM)).

Contact Printing by Robotic Systems. The spots of oligonucleotides were fabricated using a Scienion S3 spotter (Scienion AG, Berlin, GER). Spots of 350 pL were dispensed using the piezo noncontact printing system in a 16 × 8 array. Flat, 2- to 3-mm-thick stamps were glued to a glass microscope slide, oxidized, and used as substrates for PNA spotting. The PNA samples used for fabrication were prepared in water at a concentration from 1 to

(17) Tedeschi, T.; Sforza, S.; Corradini, R.; Marchelli, R. *Tetrahedron Lett.* **2005**, *46*, 8395.

(18) Thomson, S. A.; Josey, J. A.; Cadilla, R.; Gaul, M. D.; Hassman, C. F.; Luzzio, M. J.; Pipe, A. J.; Reed, K. I.; Ricca, D. J.; Wiethe, R. W.; Noble, S. A. *Tetrahedron* **1995**, *51*, 6179.

50 μM. Fifteen minutes after being spotted, the stamps were used for multiple microcontact printing. Samples were hybridized as described before and imaged using an Olympus IX71 fluorescence microscope.

Melting Experiments. The samples for the melting experiments were prepared by printing the PNA, hybridizing the PNA with a 1 μM solution of TAMRA-labeled oligonucleotide, and washing the slides with SSC at room temperature. The samples thus prepared were immersed in a 10 nM solution of the same oligonucleotide in SSC buffer containing 0.1% SDS (in order to prevent wash out from the surface during imaging) and imaged using a Carl Zeiss LSM 510 scanning confocal microscope equipped with a thermostat.

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