

# Versatile derivatisation of solid support media for covalent bonding on DNA-microchips

Markus Beier\* and Jörg D. Hoheisel

Functional Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 506, D-69120 Heidelberg, Germany

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## ABSTRACT

A chemistry was developed that permits on DNA-arrays both the covalent immobilisation of pre-fabricated nucleic acids—such as oligonucleotides, PCR-products or peptide nucleic acid oligomers—and the *in situ* synthesis of such compounds on either glass or polypropylene surfaces. Bonding was found to be stable even after some 30 cycles of stripping. Due to a dendrimeric structure of the linker molecule, the loading can be modified in a controlled manner and increased beyond the capacity of glass without negative effects on hybridisation efficiency. Also, the chemistry warrants the modulation of other surface properties such as charge or hydrophobicity. Preferentially, attachment of nucleic acids takes place only via the terminal amino-group of amino-modified oligonucleotides or the terminal hydroxyl-group of unmodified molecules so that the entire molecule is accessible to probe hybridisation. This derivatisation represents a support chemistry versatile enough to serve nearly all current forms of DNA-arrays or microchips.

## INTRODUCTION

DNA-chip technology is a synonym for the transformation of sequence information that yields from the various genome projects into a requisite of functional analyses. While the basic technique of arraying nucleic acids at high density is not new (1,2), more recent advances in synthesis chemistry or the miniaturisation of administration processes (3–6) have led to a surge in the number of applications (reviewed in 7). Currently, two support media are being used most for DNA-arrays—nylon filter and glass—both of which combine beneficial and disadvantageous features.

Due to the porous structure of filter membranes, relatively large amounts of DNA can be applied to such material, resulting in strong signal intensities and a good dynamic range. This effect is partly offset, however, by the higher background, produced mainly by the very same structural characteristic—the large surface area per spot—responsible for the high loading capacity. Filter arrays can be re-used frequently, because the DNA sticks to the nylon surface, after some initial losses of up to 50% (8). On

the other hand, much DNA is required for filter arrays production, since spot sizes cannot be reduced to a level possible with glass or other non-porous media.

This ability of miniaturising the array dimensions, in combination with chemical inertness and low intrinsic fluorescence, are the main advantages of glass (5,6,9) and polypropylene (10,11) permitting high probe concentrations even from small samples. Because of the planar surface structure, however, the loading capacity is sometimes a limiting factor. This was sought to be overcome by applying acrylamide gels to the glass for an increased surface (12,13) mimicking the filter structure; although controllable in terms of pore size, this method nevertheless introduces again some of the disadvantages of nylon membranes. A negative aspect of plain glass support is also the limitation in the number of experiments that can be done with a single DNA-chip. Many systems reported to date (5,6) permit only single usage, thus even preventing proper quality control on the very chip that is to be used in the actual experiment; re-usability of microchips also eliminates from experimentation the variance between presumably identical chips, which significantly affects the experimental reliability of chip-based analyses.

In this manuscript, the synthesis of a very flexible linker system on glass and polypropylene is reported that enables both the covalent immobilisation of nucleic acid compounds—PCR products, oligonucleotides and peptide nucleic acid (PNA)-oligomers—and the *in situ* synthesis of oligomers. The process allows for a modulation of surface properties with respect to hydrophobicity and charge. Since a dendrimeric linker system is being used, the loading capacity can be increased in a controlled manner. This is especially important to glass support, on which the loading usually is much less than on polypropylene.

## MATERIALS AND METHODS

All chemicals and solvents were purchased from Fluka (Germany) or SDS (France), unless stated otherwise, and used without additional purification.

### Support media

The following protocols refer to the preparation of a set of seven microscope slides or polypropylene sheets of the same dimension (18.2 × 7.6 cm). Reactions were carried out in polypropylene

\*To whom correspondence should be addressed. Tel: +49 6221 424684; Fax: +49 6221 424682; Email: m.beier@dkfz-heidelberg.de

vessels. As starting material, either commercially available non-derivatised microscope slides (Menzel-Gläser, Germany), aminoalkylsilane derivatised ones (SilanePrep; Sigma, Germany) or plasma-aminated polypropylene were used. If non-derivatised glass slides were used, silanisation was performed according to the following procedure in order to attach amino-groups at the surface.

### Silanisation of microscope slides

The slides were immersed in 10% NaOH overnight and subsequently washed with H<sub>2</sub>O, 1% HCl, again with H<sub>2</sub>O and finally methanol. After a 15 min sonication in a 3% aminopropyl-trimethoxysilane solution made in 95% methanol, the material was washed in pure methanol, then water, dried under a stream of nitrogen and baked at 110°C for 15 min.

### Synthesis of the linker-system

Synthesis was performed by repeating the following reaction steps 1 and 2 with the respective amines until the desired linker molecules were obtained.

*Step 1: acylation reaction.* The aminated glass slides or polypropylene sheets were incubated for 2 h in a solution made of 192 mg (1 mmol) 4-nitrophenyl-chloroformate and 171 µl (1 mmol) diisopropylethyl-amine (DIEA) in 30 ml anhydrous dichloroethane; instead of 4-nitrophenyl-chloroformate 81 µl (1 mmol) acryloylchloride could also be used. Subsequently, the supports were thoroughly washed with dichloroethane and dried.

*Step 2: reaction with amine.* The acylated material was incubated overnight with 1 mmol of the appropriate amine in 30 ml anhydrous, amine-free dimethylformamide (DMF); in case of acylation with acryloylchloride, the reaction time was extended by another 24 h. Afterwards, the slides were extensively washed with DMF, methanol and acetone before being dried.

The following amines (1 mmol) were used: tetraethylen-pentamine (223 µl); 1,4-bis-(3-aminopropoxy)butane (213 µl); 4-aminomethyl-1,8-octadecylamine (187 µl); 4,7,10-trioxa-1,13-tridecylamine (219 µl); N,N-dimethyl-1,6-hexadecylamine (173 µl); 2-(2-aminoethoxy)ethanol (100 µl); jeffamine 130 (242 µl); 3-amino-1,2-propanediol (7 µl).

*Quality assessment by bromphenol blue staining.* For control of reaction efficiency, aminated polypropylene strips were added to the reaction. After every reaction step, part of a control strip was removed, washed with DMF and subjected to a 0.05% (w/v) bromphenol blue solution in 2 ml DMF for 2 min. After subsequent washes in pure DMF and ethanol, the colour intensity was used to calculate the number of amino groups blocked during acylation or newly introduced with the amines.

### In situ synthesis of oligomer-arrays

Photo-controlled *in situ* oligonucleotide synthesis was performed with a custom-built DNA-chip-synthesiser (14) using a 100 W high pressure mercury lamp (5 min irradiation at 365 nm per cycle). Surface-bound oligonucleotides were synthesised by standard photolithographic procedures (14) using phosphoramidites protected either by 5'-O-((α-methyl-2-nitropiperonyl)oxy)carbonyl (MeNPOC) groups (5) or 5'-O-[2-(2-nitrophenyl)-propyloxy-

carbonyl] (NPPOC; 15). For the latter, the procedure was modified from the original protocol: coupling was done with 75 mM phosphoramidite for 150 s, followed by a 3 min oxidation with 50 mM iodine in a solution made of acetonitrile, pyridine and water mixed 7:1:2; irradiation took place in 50 mM piperidine in acetonitrile.

Alternatively to light-controlled reactions, oligonucleotide synthesis was carried out on the chip directed by a channel-system (9) using the high-yielding phosphoramidite chemistry described in detail earlier (11). PNA-synthesis was done as described (16) using the automated SPOT synthesiser (ABIMED, Germany).

### Activation of the surface for the immobilisation of nucleic acids

Activation of the surfaces was carried out by using either of the following reagents.

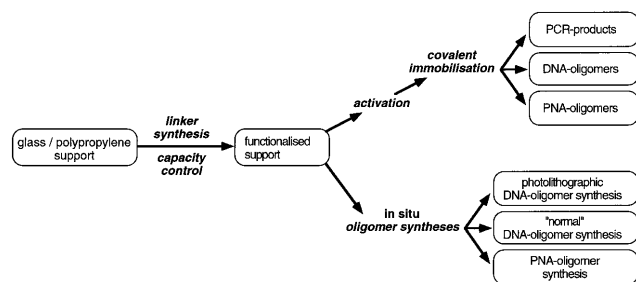
*Activation by phenylendiisothiocyanate (PDITC).* The amino-functionalised glass slides or polypropylene sheets were reacted for 2 h with 192 mg PDITC (1 mmol) in 40 ml of a 10% solution of anhydrous pyridine in DMF. Subsequently, they were washed with DMF and dichloroethane and dried under a stream of nitrogen.

*Activation by disuccinimidylcarbonate (DSC) or disuccinimidyl-oxalate (DSO).* Activation with 256 mg (1 mmol) DSC and 513 ml (1 mmol) diisopropylethylamine in 20 ml anhydrous acetonitrile took place for 4 h; alternatively to DSC, 284 mg DSO (1 mmol) were used with the same success. Washing was done as above.

*Activation by dimethylsuberimidate (DMS).* Activation was done by incubating the support material for 1 h in 40 ml saturated solution of NaHCO<sub>3</sub> containing 273 mg (1 mmol) dimethyl-suberimidate dihydrochloride. Thereafter, washing was with water and acetone followed by drying under a stream of nitrogen.

### Spotting onto activated support surfaces

Volumes of 1–100 nl of an aqueous nucleic acid solution (oligodeoxyribonucleotides, PCR-products, PNA-oligomers) at a concentration of 0.01–1 µM were placed onto the activated support media. Spotting was performed using either a piezo-driven dispensing system (Nanoplotter, GeSiM, Germany) or a pin-tool based spotting robot (BioGrid, BioRobotics, UK). Dependent on the type of surface-activator, different basic solutions of the nucleic acid were employed: PDITC, DSC and DSO: water, 1% diisopropylethylamine in water, 1% N-methylmorphine in water, 1% N-methylimidazole in water, saturated NaHCO<sub>3</sub>-solution; PDITC and DMS: water and Tris-buffer or either solution containing 1% diisopropylethylamine, 1% N-methylmorphine or 1% N-methylimidazole, respectively. After administration of the nucleic acids, the slides were incubated overnight in a humid chamber at 37°C and afterwards washed with H<sub>2</sub>O and methanol. Then, the surface was deactivated by a 2 h treatment with a solution made of 6-amino-1-hexanol (50 mM) and diisopropylethylamine (150 mM) in DMF. Alternatively to 6-amino-1-hexanol, 1,3-diaminopropane, 3-amino-1-propanol or 1-aminopropane were employed in the same concentration as above. Finally, the DNA-arrays were washed with DMF, acetone, water and dried. Storage was at 4°C.



**Figure 1.** Basic scheme of the functionalisation of glass or polypropylene supports for the generation of DNA-arrays by *in situ* syntheses or covalent immobilisation of molecules.

## Hybridisation to DNA-arrays

For the hybridisation of oligonucleotides, a 200 nM solution of fluorescently-labelled oligomer probe in 600 mM NaCl, 60 mM Na-citrate, 7.2% (v/v) Na-sarcosyl (SSARC-buffer) was used. PCR-products were labelled by employing 5'-Cy3- or 5'-Cy5-labelled primers (Ark Scientific, Germany or Interactiva, Germany) at standard conditions. Usually, a portion of a 100  $\mu$ l reaction was directly used without further modification. Hybridisation on microscope slides, or polypropylene material of the same size, was carried out under a coverslip after the application of 30–75  $\mu$ l of solution. The hybridisation temperature was determined by the type of molecule attached to the chip surface: 8mer oligonucleotides, 4–14°C; 10–20mer oligonucleotides, 20°C; PCR-products, ambient temperature to 65°C. Oligomer hybridisations were washed with cold SSARC-buffer for ~30 s. PCR-products were washed in the same buffer at 40–65°C for 5 min. After being dried under a stream of nitrogen, the DNA-arrays were scanned using a GeneArray 3000 system (General Scanning, USA) at appropriate sensitivity levels of the photomultiplier (PMT).

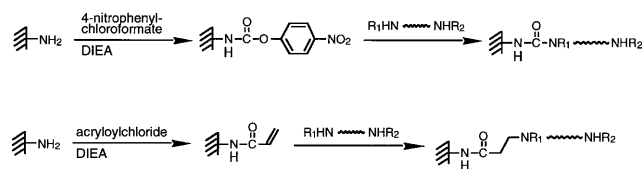
## Stripping procedure

For re-use, DNA-chips were incubated in stripping buffer [2.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1% (v/v) SDS] at 95°C for 30 s. Typically, one to two subsequent treatments gave good results. The procedure had to be extended or repeated only in case of a strong positive charge of the solid support. The DNA-chips were washed with water to remove salt and dried for storage. Usually, each DNA-chip was checked by fluorescence-scanning for complete probe removal prior to the next hybridisation.

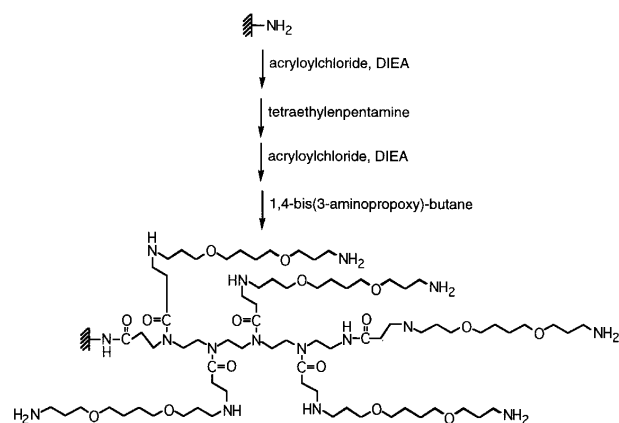
## RESULTS

### Rationale of linker design

The anticipated linker system had to meet several objectives. First and foremost, any bonding had to be covalent for frequent re-use of the arrays. Second, the system should be versatile enough to be used for all current types of array production, *in situ* syntheses as well as immobilisation of pre-fabricated molecules, and to fit the chemistries employed (Fig. 1). Third, the surface properties, such as loading capacity and charge, should be adaptable to permit optimal hybridisation results. Finally, bonding had to occur only via a terminal functional group of the attached molecule, prerequisite especially for an effective hybridisation to arrayed oligonucleotides.



**Figure 2.** Linker synthesis on aminated support. By an iterative process of acylation and reaction with various amines, linker structures of different complexity and diverse inherent characteristics can be synthesised.



**Figure 3.** Dendrimeric linker structure created by a four-step reaction as indicated. The number of reactive sites for the attachment of nucleic acids is increased by a factor of 10 by this procedure. It should be noted that not one unique product is generated by this reaction sequence, but a mixture of products; only one of them is depicted here.

Synthesis of the type of linker system described here takes place by a serial application of two reactions, a process that is repeated until the desired linker-type is created. The first reaction is an acylation of a surface-bound amino-group with an acid-chloride, either 4-nitrophenyl-chloroformate or acryloylchloride (Fig. 2). Then, the acetylated support is reacted with an amine. By varying the amine, the character of the surface can be modulated. A bis-amine results in a linear system, while a polyamine produces a branched, dendrimeric structure. Therefore, the loading of a given support could be increased in a very effective way by reacting polyamines. Ideally, the loading should be increased by a factor of 5 with tetraethylenpentamine being the amine component on an acryloylated surface (e.g. Fig. 3) and 4-fold when 4-nitrophenyl-chloroformate had been used as acylating agent. Since polyamines contain both primary and the slightly more nucleophilic secondary amino-functions, however, not only one unique product is generated in such a reaction but a mixture of compounds.

The number of amine-functions incorporated during the synthesis process also controls the number of positive charges eventually present at neutral pH. Dependent on the type of DNA-array, this could strongly influence its performance. For arrays consisting of short oligonucleotide sequences, it would be beneficial to have positive charges promoting hybridisation. On the other hand, a positively charged surface could cause serious difficulties in stripping off long probe-fragments, because of their strong electrostatic interaction with the support. Another factor that could be influenced during linker-synthesis is the hydrophilic

or hydrophobic character of the DNA-chip. Incorporation of hydrophilic amines like 1,4-bis-(3-aminopropoxy)butane results in a more hydrophilic character of the surface, hence facilitating the approach of a hydrophilic DNA-probe molecule.

### Chemistry of linker synthesis

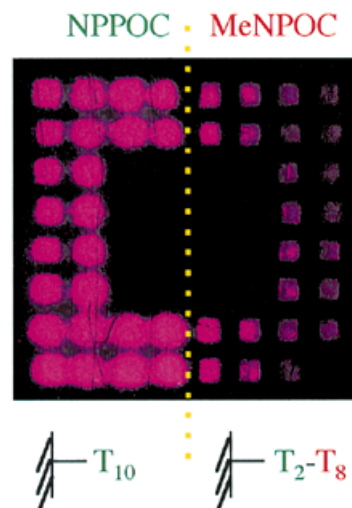
The strategy outlined above permitted the design of many different linkers. Screening large numbers of options led to the compound combinations listed in Table 1. While many linkers were well suited for either the immobilisation of nucleic acids or an *in situ* synthesis, one system (no. 21 in Table 1) especially presented itself as extremely versatile, yielding excellent results under all conditions tested. This linker was produced on aminated surfaces by subsequent reactions of acryloylchloride, tetraethylen-pentamine, acryloylchloride and 1,4-bis-(3-aminopropoxy)butane (Fig. 3). Reaction with tetraethylene-pentamine multiplied the surface loading capacity by a factor of 5. 1,4-bis-(3-aminopropoxy)butane as the second amine-component increased the distance to the surface by an additional 14 atom hydrophilic spacer and the loading capacity was raised another 2-fold.

**Table 1.** Linker systems

No.	Linker System	glass	PP	No.	Linker System	glass	PP
1	P - tepa	x		16	A - bapb	x	
2	P - dmhd	x	x	17	A - jeff	x	
3	P - bapb	x		18	A - amod - A - dmhd	x	x
4	P - jeff	x		19	A - amod - A - bapb	x	
5	P - amod - P - dmhd	x	x	20	A - bapb - A - bapb	x	
6	P - amod - P - bapb	x		21	A - tepa - A - bapb	x	x
7	P - bapb - P - bapb	x		22	A - tepa - A - amod	x	x
8	P - tepa - P - bapb	x	x	23	A - tepa - A - apd	x	
9	P - tepa - P - amod	x	x	24	A - tepa - A - ttd	x	x
10	P - tepa - P - dmhd	x	x	25	A - tepa - A - aee	x	
11	P - tepa - P - apd	x		26	A - tepa - A - ah	x	
12	P - tepa - P - ttd	x	x	27	A - tepa - A - tepa - A - apd	x	
13	P - tepa - P - aee	x					
14	P - tepa - P - ah	x					
15	P - tepa - P - tepa - P - apd	x					

Linker systems are listed which were synthesised on glass or polypropylene (PP) by consecutive reactions of the following reagents: A, acryloylchloride; P, 4-nitrophenyl-chloroformate; aee, 2-(2-aminoethoxy)ethanol; ah, 6-amino-hexanol; amod, 4-aminomethyl-1,8-octadecylamine; apd, 3-amino-1,2-propanediol; bapb, 1,4-bis-(3-aminopropoxy)butane; dhd, N,N-dimethyl-1,6-hexadiamine; jeff, jeffamine 130; tet, tetraethylen-pentamine; ttd, 4,7,10-trioxa-1,13-tri-decandiamine.

Quantification as above was made possible by monitoring the reaction steps by bromphenol blue staining. For quality assessment, an aminated polypropylene strip was added to each synthesis as a control. While amino functions were blocked during acylation, new amino-functions were introduced by the reaction with amines. After each step, a part of the control strip was removed and subjected to the staining procedure indicating the reactive amino-groups. This control was carried out routinely but usually checked visually only. The blue colour reaction also intensified with the build-up of the dendrimeric linker structure.



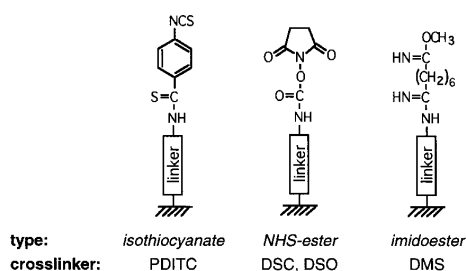
**Figure 4.** Oligonucleotide-chip synthesis. An array of d(T<sub>10</sub>) sequences was generated by photolithographic *in situ* synthesis on a microscope slide derivatised with linker no. 21. Either 10 consecutive cycles with NPPOC-protected phosphoramidites were performed (left) or a mixed reaction of two cycles with NPPOC- and eight cycles with MeNPOC-phosphoramidites (right). The oligomer patches were made visible by hybridisation with probe d(A<sub>16</sub>), labelled with dye Cy5 at its 5'-end.

### *In situ* oligomer synthesis

For *in situ* synthesis of oligomers, no further derivatisation was necessary. The terminal amino (or hydroxyl) groups of the linker molecules served directly as starting points for synthesis. Oligonucleotides were synthesised either by a modified phosphoramidite chemistry described in detail earlier (11) or a photolithographic process. For 5'-protection during the latter, photocleavable 2-(2-nitrophenyl)propyloxycarbonyl-groups (NPPOC-groups; 15) were employed in comparison to the 5'-O-(( $\alpha$ -methyl-2-nitropiperonyloxy)carbonyl)-groups (MeNPOC-groups; 5,17). The NPPOC-chemistry represents a new class of rapidly detachable photoprotecting groups of significantly higher coupling efficiencies (M.Beier and J.D.Hoheisel, manuscript in preparation). Enhanced photo-cleavage takes place via a base-assisted  $\beta$ -elimination process. Photo-controlled synthesis was performed in a custom-built instrument on slides placed in a flow cell (14). The only manual operation involved was the exchange of the masks. Figure 4 shows a typical array containing dT<sub>10</sub>-sequences synthesised on linker system no. 21 (Table 1) with mixed NPPOC- and MeNPOC-chemistries. The obvious differences in signal intensity were due to the improved yield of the NPPOC-based synthesis protocol. In all cases, loading capacities and quality of the synthesised oligonucleotides were as expected.

### Immobilisation of nucleic acid compounds

**Surface activation.** To attach nucleic acid compounds covalently to a solid support, a chemical bond had to be formed between a functional group of the biomolecule and the amino-group of the linker. For bond formation, either of the groups had to be transferred into an activated state. In our hands, best results were obtained by activating the solid phase surface, thereby also preventing any cross-reaction between the nucleic acids prior to



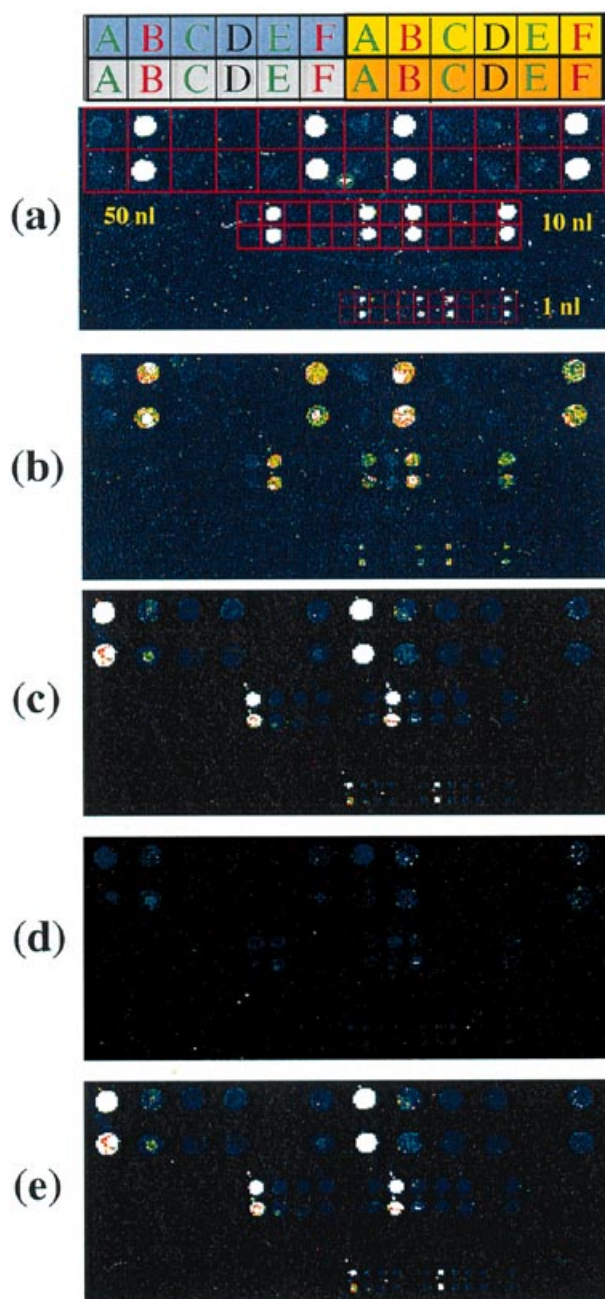
**Figure 5.** Activation of the microchip surface for the immobilisation of pre-made DNA-molecules; the active groups formed by the respective crosslinkers are shown.

immobilisation. The homobifunctional crosslinking agents phenyl-endiisothiocyanate (PDITC), disuccinimidylcarbonate (DSC), disuccinimidyl oxalate (DSO) and dimethylsuberimidate (DMS)—well known in bioconjugate chemistry (18)—were used to convert the chip-bound amino-groups into reactive isothiocyanates, N-hydroxysuccinimidyl-esters (NHS-esters) or imidoesters, respectively (Fig. 5). Activation with PDITC (19), DMS, DSC and DSO was found to be best for the covalent attachment of oligonucleotides and PNA-oligomers, while for immobilisation of amino-linked PCR-fragments crosslinking with PDITC or DMS was superior, since they are less labile to the Tris buffer reagent present in the PCR-mixtures. Routinely, PDITC and DMS were used for the applications described here, for their high efficiency irrespective of the type of compound added.

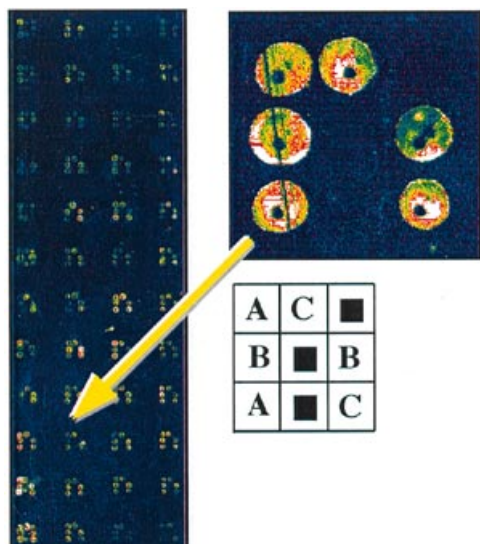
**Probe coupling.** Prior to spotting, usually a base was added to the nucleic acid solution, since all crosslinking agents used have their optimal reactivity in a basic milieu. Because of its non-nucleophilic character, a base like diisopropylethylamine was employed, since it does not compete for the reactive sites on the chip. However, immobilisation worked also at neutral pH, i.e. in water (Fig. 6a).

Small volumes of the nucleic acid solution were initially placed onto the activated support by hand-held glass-capillaries. For actual chip production, convenient spotting robots equipped with very fine pin-tools (BioGrid, BioRobotics, UK) or a piezo-driven dispensing system (Nanoplotter, GeSiM, Germany) were used. Typically, volumes between 1 and 50 nl were transferred, but even below 1 nl good signal intensities could be detected upon hybridisation. Because these small droplets evaporated quickly after application, the reactions were run to completion by incubating the DNA-arrays in a humid chamber at 37°C for several hours subsequent to the administration of all spots. The chips were then washed with water and methanol followed by a deactivating step, quantitatively blocking all still reactive functions on the surface. By this, any non-specific binding of DNA at later stages, causing high background (19), was prevented.

**Variation of the surface hydrophobicity.** By choosing different amines for the deactivation, the DNA-chips could be modified with respect to their hydrophilic character or the number of charges present after the nucleic acids had been immobilised. This was tested by comparing the hybridisation behaviour of DNA-arrays when 1-aminopropane, 3-amino-1-propanol and 1,3-bis-aminopropane were employed in the deactivation reaction. Clearly, DNA-arrays being deactivated with the more hydrophobic propylamine were outperformed by the other more hydrophilic



**Figure 6.** Hybridisation to immobilised oligonucleotides. Arrays were made by immobilisation of oligonucleotides on microscopic slides activated by PDITC. Six different oligonucleotides (A–F) were spotted by piezo-pipetting in  $12 \times 2$  patterns using four different solvents: H<sub>2</sub>O (blue shaded area); 1% diisopropylethylamine in H<sub>2</sub>O (yellow); 1% N-methylimidazole in H<sub>2</sub>O (grey); 1% N-methylmorpholine in H<sub>2</sub>O (orange). Three identical arrays of different spot sizes were generated; spotted volumes were 50, 10 and 1 nl; spot diameters ~0.8, 0.5 and 0.2 mm. Oligomers in red letters (B and F) in the pipetting scheme were directly labelled with Cy5 at their 5'-end; all green lettered oligomers (A, C and E) were attached via a 5'-amino-group; oligomer D had hydroxyl-groups at both 3'- and 5'-ends. Results obtained on one single chip are shown: (a) the slide directly after spotting; only the 5'-Cy5-labelled controls (B and F) were detected (PMT 100%); (b) signals obtained after two hybridisation and stripping events (PMT 100%); (c) third hybridisation using 5'-Cy5-d(GCAGTGGTGGAGAAAGAGTAAC) complementary to position A (PMT 70%); (d) signal after sixth probe stripping (PMT 100%); (e) seventh hybridisation using the same probe as in (c) (PMT 70%).



**Figure 7.** Probe hybridisation to array of PCR-fragments. 144 different 5'-amino-linked PCR-fragments were placed by a pin-tool on a microscope slide using linker system no. 21 activated by DMS. The DNA was spotted in a  $3 \times 3$  pattern with each PCR-product being present twice as indicated. Hybridisation was done with a 20mer 5'-Cy5-labelled oligonucleotide complementary to a tag sequence common to all PCR-products.

amines (3-hydroxy-propylamine; 1,3-bis-aminopropane). Routinely, 6-aminohexanol was used for the deactivation reaction.

**Coupling efficiencies.** DNA with various modifications at either 5'- or 3'-end was used in spotting (5'-amino, 5'-Cy5, 3'-amino-5'-Cy5 and unmodified oligonucleotides; amino-linked and ordinary PCR-products; PNA-oligomers). All could be successfully attached to the activated support. However, there were significant differences in the stability of the bonding (Fig. 6). Only the 5'-amino oligonucleotides were found to be attached covalently for more than two hybridisation and stripping events, while oligomers attached via a hydroxyl-function (e.g. Cy5-labelled oligomers B and F in Fig. 6) all but disappeared. From this difference, it could be concluded that only little covalent linkage occurred via any of the internal amino-groups of a DNA-molecule. Because of this terminal attachment of prefabricated oligonucleotides, their entire sequence was accessible for probe hybridisation.

The same effect could be seen for PCR-products. In Figure 7, a typical hybridisation to different PCR-fragments is shown. The sequence used was complementary to the most 5'-located bases of one strand of the PCR-fragments. In comparison to other, more internally binding sequences, no difference in hybridisation was observed.

Attachment of PNA-oligomers worked well in the same way (Fig. 8) but giving increased signal intensities compared to oligonucleotide sequences due to their higher binding affinity in hybridisation. Even at low sensitivity of the PMT, hybridisation to the lowest concentration spotted was easily detected. Although the optimal pH of PDITC-activation is at pH 8–10, best immobilisation results with PNA-oligomers were obtained, when a less basic solvent (1% N-methylimidazole in water) was used. This effect was probably caused by the better solubility of PNA at neutral or acidic pH.

## Hybridisation experiments

Hybridisation to arrays made of both oligomers or PCR-products indicated no apparent effect of the dendrimeric structure to the kinetics of hybridisation; both probe annealing and stripping worked fine. Also, no formation of aggregates between linkers or the attached biomolecules could be detected. As compared to porous material, such as filters, no non-specific attachment of the hybridisation probe occurred; any material remaining after insufficient washing could be removed by an additional step of rinsing the arrays. The reduced background alone improved sensitivity considerably, with the effect being enhanced further by the ability of using increased probe concentrations.

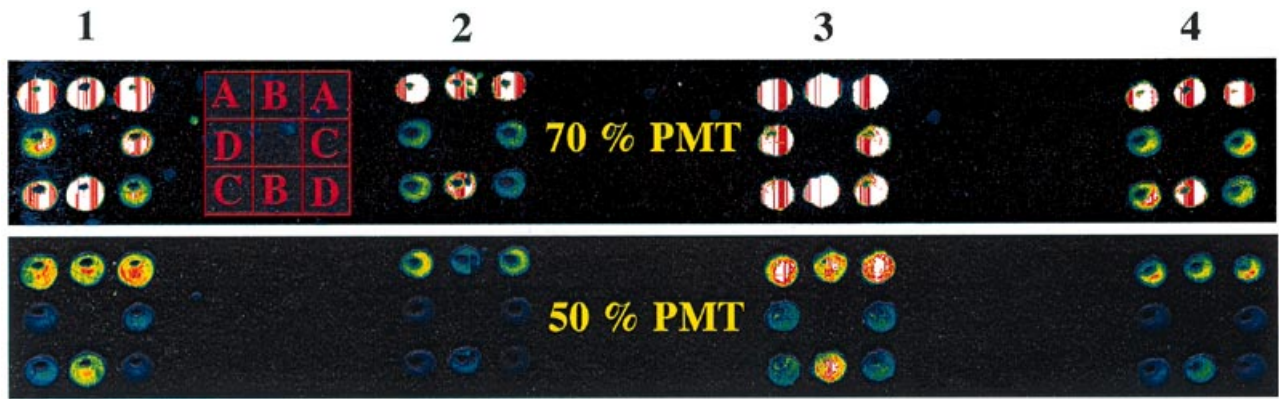
**Re-usability.** One imperative objective of our work was the re-usability of the DNA-arrays generated by our method. By design, all DNA-fragments, whether generated by *in situ* synthesis or immobilisation, were covalently bound to the surface. This was confirmed by subjecting such chips to the conditions of standard hybridisation and stripping procedures carried out at neutral pH. In every case, the DNA-arrays withstood more than seven actual consecutive cycles of hybridisation and stripping without any significant loss of signal intensity upon imaging (Fig. 6). Furthermore, between real hybridisation experiments DNA-arrays were immersed in the stripping solution and mounted onto a custom-built DNA-chip-thermocycler. Even after 30 cycles of conditions identical to those of hybridisation and stripping (1 min at 95°C, 5 min at 25°C), no apparent loss of signal was observed (Fig. 9).

## DISCUSSION

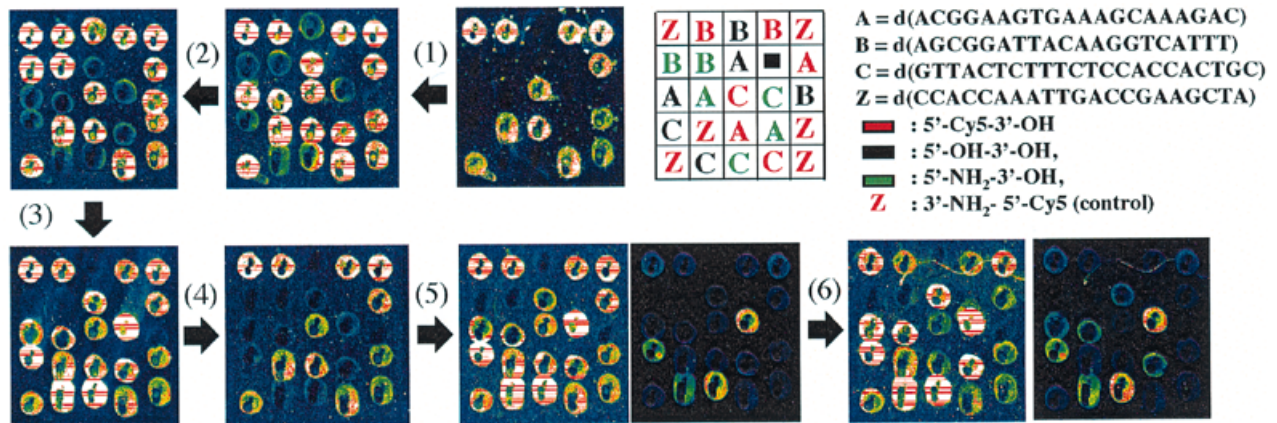
DNA-microchip technology will have an enormous impact on analyses in biology, molecular medicine and many other areas. Although the method's real width and depth of effecting these fields is still far from being reached, current studies already demonstrate clearly the tremendous potential and consequences (e.g. 20–23). While many of the elementary procedures of microchip technology have been established, quite some headway is still to be made towards improved quality and regular application. The development of the versatile linker system described here adds to this end.

For an ideal comparison of two specimens, exactly the same experimental conditions had to be applied. While this will never be accomplished because of the many factors involved, re-use of the very microchip for several times eliminates one critical factor of variation between analyses. By these means, accuracy levels could be raised by the implementation of quality assessment procedures which are otherwise impossible. In addition, surface conditions such as loading capacity, charge and hydrophobicity could be modulated to meet the specific requirements of the relevant experiment best. Although not all combinations of linker-synthesis possible on the basis of the described chemistry were tested, our screening identified several suitable for at least one of the applications outlined in Figure 1. Of these, especially one systems yielded good results with all forms of DNA-arrays used in our laboratory. In addition, the immobilisation of thiol-modified nucleic acids should be possible, although this process has not been tested yet.

Most data presented here were obtained on glass rather than polypropylene, since glass is the more challenging surface with respect to loading capacity and thus signal intensities. In all cases,



**Figure 8.** Hybridisation to PNA-oligomer. Approximately 2 nl of the 18mer PNA-molecule Lys-Lys-OTCTGAGAGCCCACTCGGAO-Lys-Lys were spotted at different concentrations: A, 1  $\mu$ M; B, 0.5  $\mu$ M; C, 0.1  $\mu$ M; D, 0.01  $\mu$ M, using different basic solvents (1, saturated  $\text{NaHCO}_3$ ; 2, 1% diisopropylamine in  $\text{H}_2\text{O}$ ; 3, 1% N-methylimidazole in  $\text{H}_2\text{O}$ ; 4, 1% N-methylmorpholine in  $\text{H}_2\text{O}$ ) onto PDITC-activated microscope slides containing linker no. 21. Hybridisations were with a complementary Cy5-labelled DNA-probe.



**Figure 9.** Multiple use of DNA-arrays. Four oligonucleotides (A–C and Z) were spotted in a  $5 \times 5$  dot pattern ( $3 \times 3$  mm) onto a microscope slide derivatised with linker no. 21 activated by PDITC; spot sizes were 0.5 mm. In part, the oligomers were modified with Cy5 and/or amino-groups as indicated. (1) Hybridisation with oligonucleotide complementary to A (PMT 100%); (2) stripping followed by hybridisation with oligonucleotide complementary to B (PMT 100%); (3) stripping followed by hybridisation with oligonucleotide complementary to C (PMT 100%); (4) 12 cycles of stripping and hybridisation conditions; (5) hybridisation with oligonucleotide complementary to C (left: PMT 100%, right PMT 65%); (6) 15 cycles of stripping and hybridisation conditions followed by hybridisation with oligonucleotide complementary to C (left, PMT 100%; right, PMT 65%).

however, similar results were obtained on polypropylene. Using fluorescence probe-labelling, glass performed better than polypropylene because of less background. While the flexibility of polypropylene proved advantageous for handling, it caused problems during the scanning process in a confocal scanning device. Also during *in situ* syntheses, glass was slightly superior because of its optical features and the lack of any swelling as observed with polypropylene. However, in ongoing transcriptional profiling experiments using arrays containing immobilised PCR-products representing the entire gene repertoire of yeast and *Arabidopsis*, polypropylene membranes proved their worth for the daily handling processes involved in such analyses.

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