Dendrimer-Activated Solid Supports for Nucleic Acid and Protein Microarrays

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The generation of chemically activated glass surfaces is of increasing interest for the production of microarrays containing DNA, proteins, and low-molecular-weight components. We here report on a novel surface chemistry for highly efficient activation of glass slides. Our method is based on the initial modification of glass with primary amino groups using a protocol, specifically optimized for high aminosilylation yields, and in particular, for homogeneous surface coverages. In a following step the surface amino groups are activated with a homobifunctional linker, such as disuccinimidylglutarate (DSG) or 1,4-phenylenediisothiocyanate (PDITC), and then allowed to react with a starburst dendrimer that contains 64 primary amino groups in its outer sphere. Subsequently, the dendritic monomers are activated and crosslinked with a homobifunctional spacer, either DSG or PDITC. This leads to the formation of a thin, chemically reactive polymer film, covalently affixed to the glass substrate, which can directly be used for the covalent attachment of amino-modified components, such as oligonucleotides. The resulting DNA microarrays were studied by means of nucleic acid hybridization experiments using fluorophor-labeled complementary oligonucleotide targets. The results indicate that the novel dendrimer-activated surfaces display a surface coverage with capture oligomers about twofold greater than that with conventional microarrays containing linear chemical linkers. In addition, the experiments suggest that the hybridization occurs with decreased steric hindrance, likely a consequence of the long, flexible linker chain between the surface and the DNA oligomer. The surfaces were found to be resistant against repeated alkaline regeneration procedures, which is likely a consequence of the crosslinked polymeric structure of the dendrimer film. The high stability allows multiple hybridization experiments without significant loss of signal intensity. The versatility of the dendrimer surfaces is also demonstrated by the covalent immobilization of streptavidin as a model protein.

KEYWORDS:

dendrimers · DNA · immobilization · microarrays · surface chemistry

Introduction

DNA microarrays are of tremendous importance for various applications regarding nucleic acid analyses, such as the monitoring of mRNA expression, the re-sequencing of DNA fragments for the genotyping of single-nucleotide polymorphisms, and the detection of viruses and other pathogens.[1] For instance, DNA microarrays have recently been applied for the classification of human tumors.[1c,d] The fabrication of the microarrays can be achieved by either an on-chip synthesis using phosphoramidite-based chemistry combined with photolithographic structuring techniques,[2] or by the automated deposition of prefabricated oligonucleotides onto chemically activated supports by means of ink-jet or spotting devices. The latter approach has the potential to become the leading method of microarray preparation since it is extremely versatile and not limited to DNA chips, but can also be applied to arrays containing proteins[3, 4] and low-molecular-weight compounds.[5] For instance, antibody microrarrays are highly attractive as multianalyte immunosensors in clinical diagnostics since the miniaturization of ligand-binding assays not only reduces costs by decreasing reagent consumption, but can simultaneously exceed the sensitivity of macroscopic techniques.[4] With respect to proteom research, protein microarrays are used for high-throughput screening for protein–protein interactions, the identification of protein kinase substrates, and the identification the protein targets of small molecules.[3] High-density arrays of small molecules have been used to identify protein receptors,[5a,b] and for the rapid determination of the enantiomeric excess of tens of thousands of compounds obtained from combinatorial libraries.[5c]

Amongst the large number of solid support materials described for the production of microarrays,[6] silica is most often used due to its great chemical resistance against solvents, its mechanical stability, and its low intrinsic fluorescence properties. Moreover, the surface silanol groups of silica are sufficiently reactive to allow covalent modification using alkoxysilanes, such as aminopropyltriethoxysilane.[6a,f] It has been described that water, which is either bound to the silica surface or is present in the silylation reagent solution, hydrolyzes the alkoxysilanes to

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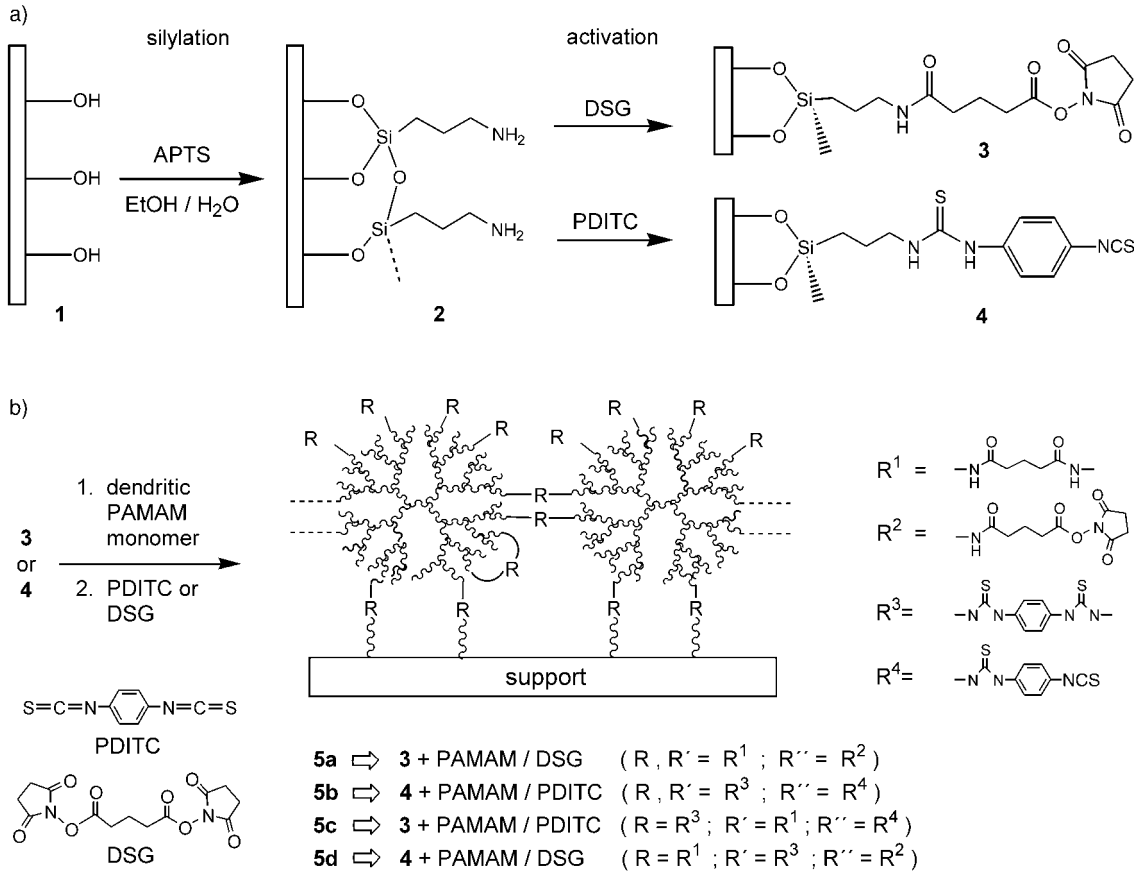
form silanols, which then intermolecularly condensate and covalently bind to the surface silanol groups, thereby generating a cross-linked silylated silica surface.[7, 8] However, since DNA microarray applications essentially require reproducible and quantifiable coupling protocols, the solid supports need to be prepared by highly standardized silylation procedures that allowing the homogeneous and efficient activation of the entire surface. Various methods have been suggested, such as silylation using water-free reaction conditions in toluene or xylene,[9] or using an additive of small amounts of water to the reaction solution in toluene[8, 9b, 10] or in alcohol as solvent.[2c, 6f, 9b] In addition to problems regarding inhomogenous surface silylation, a general drawback of the silica supports is that often only poor surface coverage with oligonucleotides can be obtained in the case of simple chemical linkers, such as glutaraldehyde- or oligoethyleneglycole-based groups.[6e, 9b, 11] In addition, such microarrays often do not withstand frequent regeneration steps. To overcome such problems, procedures have been reported to build up a three-dimensional linker system by a multistep functionalization of glass surfaces.[6a–c] Although improving the surface's capacity for the binding of oligonucleotides, these approaches require time-consuming preparations or often do not withstand harsh regeneration conditions. Beier and Hoheisel reported on dendritic linker systems for the fabrication of microarrays.[6f] However, the dendritic linkers described were generated in situ by several time-consuming modification steps, and thus, the structure of the linkers actually present on the surfaces was unclear. In addition, the surfaces revealed inhomogeneous distributions of the signal intensity within individual spots. Thus, we reasoned that the employment of prefabricated dendritic macromolecules[12] might simplify the procedure and improve the quality of supports, chemically activated for the immobilization of biomolecules.

We report here on a novel method for generating highly homogeneous, chemically activated silica surfaces that can be applied for the efficient coupling of oligonucleotides to produce DNA microarrays. First, the aminosilylation procedure was systematically optimized to yield homogeneous amino-modified surfaces (Scheme 1 a). As observed by photometric quantification the quality of the surface, and thus, the quality of the DNA microarray depends strongly on the solvent used in the silylation process. In subsequent steps, the aminosilylated surface was



Scheme 1. *Schematic representation of surface modifications. a) Generation of silica surfaces **3** and **4** containing linear linkers. Reactive hydroxy groups of the glass surface **1** condense with 3-aminopropyltriethoxysilane (APTS) to generate the amino-silylated surface **2**. Subsequently, the surface amino groups are treated with homobifunctional linker reagents DSG or PDITC to generate chemically activated surfaces **3** and **4**, respectively. These surfaces are appropriate to either directly immobilize DNA oligomers, or to serve as an anchor system for the covalent attachment of a polymeric dendrimer thin layer. Crosslinking of the homobifunctional linkers directly on the surface is omitted. b) In situ generation of polymerized dendritic surfaces **5a** – **5d**. Surfaces **3** or **4** are coupled with a starburst PAMAM dendrimer that contains 64 primary amino groups in its outer sphere, and in a second step, the dendritic macromolecules are activated and cross-linked by either DSG or PDITC. The latter reaction leads to intra- and intermolecular crosslinking, as well as to the attachment of reactive end groups suitable for the direct coupling of amino-modified DNA oligomers or proteins.*

functionalized with an in situ generated polymeric linker system, using a homobifunctional spacer, a polyamino-functionalized dendritic PAMAM starburst macromolecule, and activating and intermolecularly cross-linking the monomeric dendrimers with an homobifunctional reagent (Scheme 1 b). This procedure leads to the formation of a chemically activated polymer film, covalently attached to the glass support, which can be used directly for the covalent immobilization of amino-modified DNA oligomers. As determined by autoradiography, the surface loading is as high as 150 fmol mm^{-2}. Moreover, the dendrimer-based surfaces can be regenerated many times without significant loss in signal intensity. As an additional advantage the polarity of the dendrimer-modified surface can be modulated by using different crosslinking reagents, thereby allowing the alteration of the microarray spot's size and sharpness.

Results and Discussion

Silylation

To develop an optimized procedure for the activation of glass cover slides for immobilizing bioorganic components, such as DNA oligomers or proteins, the silylation of the silica surface was studied as an initial modification step. Two criteria of the silylation protocol were experimentally optimized to reproducibly generate high-grade surfaces that meet the demands for DNA microarray preparation. First, surface coverage with cross-linked silane needs to be highly homogeneous throughout the entire surface, thereby allowing quantitative interpretation of the hybridization signals. Second, it is particularly important to maximize the number of functional groups introduced by the silylating agent. A large number of coupling groups allows the high density immobilization of capture oligonucleotides, and thus, enables low limits of detection during hybridization analyses.

As depicted in Scheme 1 a, conventional protocols for modification of silica surfaces are often based on the initial silylation of microscope glass slides **1** using aminopropyltriethoxysilane (APTS) to obtain a amino-derivatized surface **2**,[9, 10] which is subsequently activated by using, for instance, disuccinimidyl-glutarate (DSG) or phenylenediisothiocyanate (PDITC) to obtain amino-reactive surfaces **3** and **4**, respectively. With respect to the development of optimized surfaces for DNA microarray preparation, several methods for the silylation of glass supports were compared. The influence of the organic solvent and water on the modification of the glass slides with APTS was studied under various conditions. Therefore, the silylation was carried out both under anhydrous conditions in toluene, in toluene containing a definite amount of water, as well as in defined solvent mixtures of ethanol and water. In additon, the reaction time was varied between one and twenty hours. The surface activiation was quantitatively determined by coupling with the sulfonyl chloride activated dye rhodamine B, which allowed the photometric determination of immobilized amino groups (Figure 1 a). After the covalent coupling of the dye, the absorbance of the colored slides was measured at $\lambda_{max} = 550$ nm. As shown in Figure 1 b, the immobilization capabilities of surface **2** were increased when

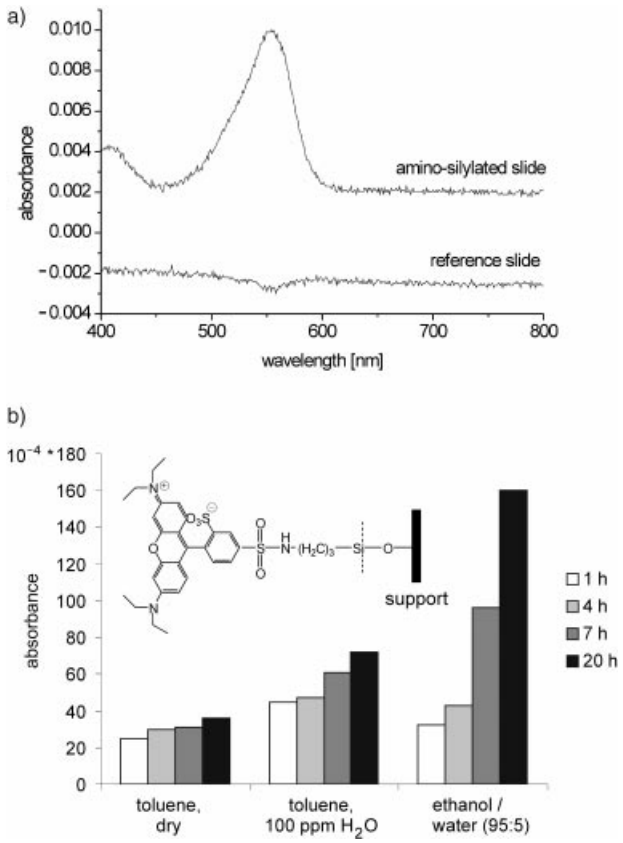


Figure 1. *Quantitative determination of amino groups attached to glass cover slides by the APTS reaction depicted in Scheme 1. a) Typical UV/Vis absorbance spectrum for the treatment of the amino-modified surface **2** with rhodamine B sulfonyl chloride. The absorbance at 550 nm indicates successful amino silylation of the glass surface. The spectrum of a reference slide without amino groups is shown for comparison. b) Quantification of the amino groups obtained by using the various silylation protocols studied (see text for details). The height of the histogramms corresponds to the relative number of amino groups, as determined by the absorbance at 550 nm, subsequent to reaction with rhodamine B sulfonyl chloride. In addition, the structure of immobilized dye is shown.*

the slides were treated for prolonged duration with the silylating reagent. A significantly larger amount of dye was coupled to the surface when water was present as a cosolvent. Presumably, this is due to an increased hydrolysis of the alkoxysilane, leading to an increased deposition of aminosilyl precipitation on the glass surface.[8] The highest immobilization efficiency was obtained when the silylation was carried out for twenty hours in ethanol/water (Figure 1 b).

To further elucidate the silylation step, the surface roughness of the silane-coated surfaces was investigated by scanning electron microscopy (SEM) and atomic-force microscopy (AFM) (Figure 2). These techniques revealed that the silylation carried out in toluene leads to highly inhomogeneous and rough surfaces (Figure 2 a). This effect is particulary strong when small amounts of water were present during the silylation procedure (Figure 2 b). Although often described in the literature,[9, 10] these conditions led to surfaces which often reveal high background and inhomogeneous surface coverages (see below). As indicated by SEM analysis, large silica depositions are inhomogeneously distributed on the chip's surface, and thus, lead to nonuniform

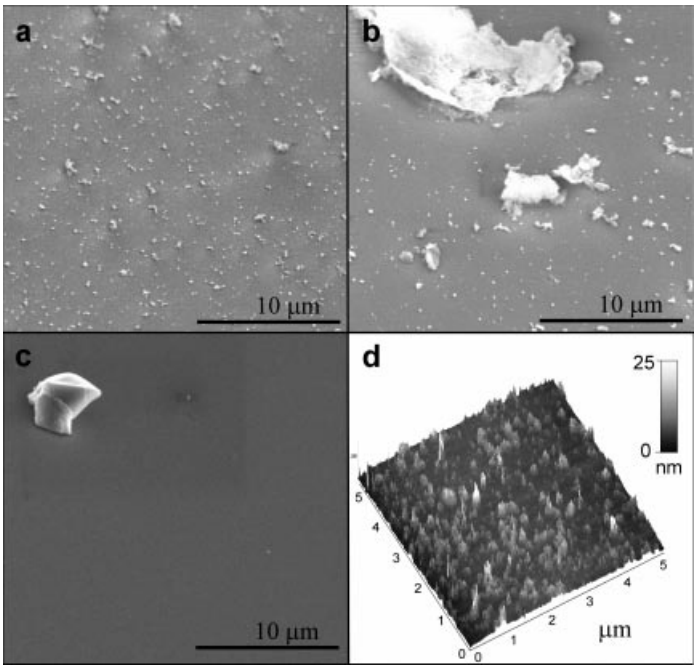


*Figure 2. SEM and AFM characterization of surfaces **2** obtained from the various silylation protocols. Glass slides were silylated under various conditions. a,b) SEM images of surfaces obtained by amino-silylation for 4 h at 80 °C in toluene under anhydrous conditions (a) or in the presence of 100 ppm water (b). Note the silica depositions on the surfaces. c) SEM image of a surface obtained by amino-silylation for 4 h at 25 °C in ethanol/water (95:5 v/v). Note the smoothness of the surface. The glass splinter is shown to prove the exact focussing of the image. d) AFM image of the surface shown in Figure 2 c, indicating a maximum surface roughness of less than 25 nm.*

intensity distributions during hybridization analyses with the DNA microarray. In contrast, silylated surfaces **2** generated in the ethanol/water solvent are very smooth (Figure 2 c). No surface structure was detected by SEM analysis, and thus, AFM was used to further characterize the surface roughness (Figure 2 d). Whereas the unmodified, freshly cleaned microscopy slides showed a maximum surface roughness below 25 nm, silylation carried out for less than four hours led to surfaces showing a roughness in the same range. This indicates that a very smooth silane coating has been generated. An increase of the silylation time again leads to silica depositions, similar to those obtained using the toluene-based solvent system (not shown).

Optimization of the linker-system

Strategy: Based on the results obtained from APTS silylation, we then systematically investigated various linker systems suitable for the covalent attachment of amino-containing components, such as DNA oligomers and proteins. The linker systems studied allowed the generation of two classes of solid supports, 1) surfaces containing conventional linear linkers, such as **3** or **4**, (Scheme 1 a), and 2) surfaces containing a polymerized dendritic coating, such as **5 a** – **d** (Scheme 1 b). In the case of the linear linker-containing surfaces, **3** and **4**, the activation of **2** with DSG or PDITC leads to the carboxylic ester or isothiocyanate-activated surfaces, respectively. These two surfaces were used as an anchor system to covalently attach the dendrimer coating, and also to serve as reference system for the surfaces **5 a** – **d**. The latter were prepared by reacting amino-modified surfaces **3** or **4** with a polyamino-functionalized dendritic PAMAM starburst macromolecule and subsequent treatment with the homobifunctional reagents DSG or PDITC.

The concept for the preparation of dendrimer-modified surfaces was based on the assumption that the starburst dendrimer with its 64 primary amino groups in the outer sphere, might be intermolecularly crosslinked by treatment with the homobifuctional reagents DSG or PDITC, after coupling of the dendritic monomers to surfaces **3** and **4**. We reasoned that the surface coating with the polymerized dendrimer film should lead to an increase in the number of reactive groups, and thus, should allow the binding of large amounts amino-derivatized oligonucleotides. Moreover, we expected that the cross-linked polymer film should improve the stability of the surface against harsh washing and regeneration conditions applied during microarray analyses. The polymeric thin-film nature of the dendrimer-modified surfaces is illustrated by the images in Figure 3. A DNA microarray based on the surface **5 b** was mechanicallly scratched after its use in hybridization analysis (see below). A cross-section analysis of the edge using AFM indicated that a homogeneous layer with a thickness of about 100 nm was formed by the dendrimer deposition procedure (Scheme 1 b).

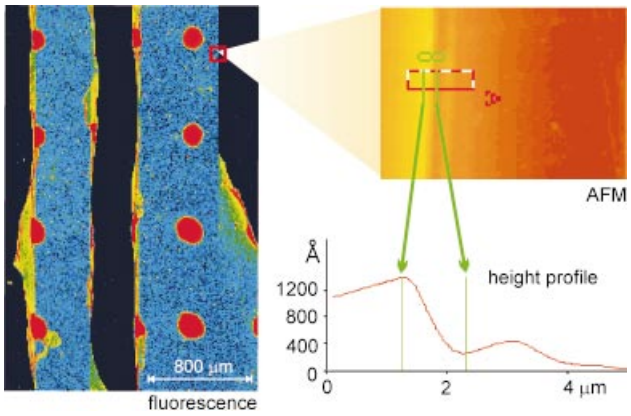


*Figure 3. Demonstration of the polymeric thin-film nature of the dendrimer-modified surface **5 b**. A DNA microarray was mechanically scratched after its use in hybridization analysis (left). The polymer-coated area appears as light blue areas containing red hybridization signals, while the scratched glass support is colored dark blue. AFM imaging was used to measure the height of the film. Cross-section analyses of the edge indicates that the polymer layer has a thickness of about 100 nm.*

Surface properties: A series of dendrimer-modified supports **5 a** – **d** (Scheme 1 b) were synthesized from preactivated surfaces **3** or **4** by using either PDITC or DSG as a homobifunctional activating reagent. All surfaces were tested in DNA microarray analyses. In particular, the surfaces were used to covalently attach an array of amino-derivatized capture oligomers, and subsequently, Cy5-labeled target oligomers were allowed to hybridize with the array-bound capture oligomers. As an initial result, the surface **5 d**, generated from the isothiocyanate surface **4** by subsequent activation with PAMAM and DSG, revealed

extremely diffuse oligomer spots as well as a very low stability against the alkaline regeneration conditions. Accordingly, surface **5d** was judged inappropriate for DNA-microarray applications and was not further characterized.

In contrast, surfaces **5a**–**c** proved to be highly suitable for the preparation of DNA microarrays. These surfaces showed different properties with respect to polarity, background signal intensity, and regeneration stability (Figure 4). As judged from the sharpness of fluorescent hybridization signals, the surface polarity of **5a** and **5b** appeared to be nearly identical. The surfaces **5a** and **5b** revealed a high hydrophobicity which led to the formation of highly symmetrical and sharp-edged spots. Spotting of 250 pL of the amino-modified capture oligomers with a piezo-driven dispensing device led to an average spot diameter of approximately 170 μm. As another advantage, the nonspecific binding of the Cy5-labeled target oligomer to surface **5a** is very weak, likely a consequence of the negativly charged carboxylic groups on the surface. This leads to high signal-to-background ratios upon fluorescence analysis. In contrast, surface **5c** showed less hydrophobic properties. The depostion of 250 pL of the capture oligomer solution leads to spots with an average diameter of approximatly 600 μm. Although this allowed very high detection sensitivity, the diffuse shape of the spots is disadvantageous for standard hybridization analyses. As indicated by the overlay of three cross-sections of different spots, obtained with different arrays, the dendrimer activation of the slides is homogeneous and reproducible (Figure 4). As shown in Figure 5, this allows highly homogeneous hybridization signal intensities on the entire surface. The high homogeneity of the spots obtained with PAMAM dendrimer coated slides is demonstrated in Figure 6. Two commercially

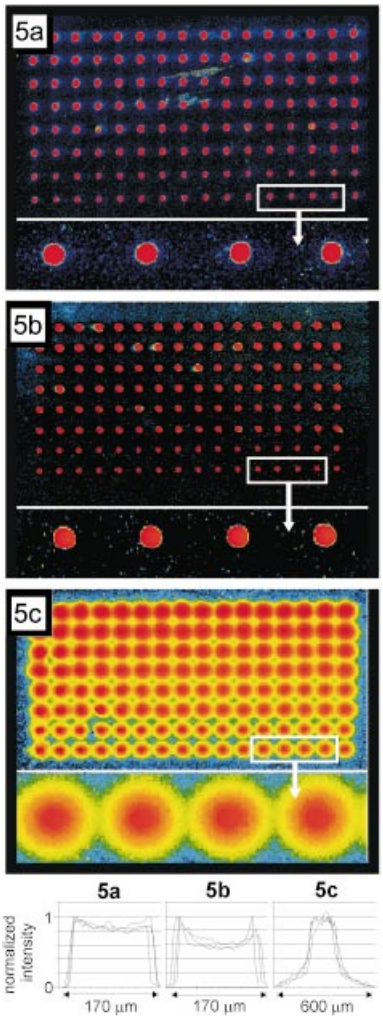


Figure 4. *Utilization of dendrimer surfaces* ***5a*** *–* ***c*** *to generate DNA microarrays. The differences in hydrophobicity of surfaces* ***5a*** *–* ***c****, leading to variations in the size and sharpness of the spots are illustrated. The arrays were prepared by depositing volumes ranging from 0.25 – 1 nL of a 5′-amino modified DNA oligomer in water onto the dendrimer surfaces. Subsequently, the arrays were hybridized with a complementary 5′-Cy5-labeled oligomer, and hybridization signals were detected with a fluorescence scanner. Overlay plots of cross-sections, obtained from various 250 pL spots of several different slides, are shown at the bottom. Note the high reproducibility of the intensity distribution within the individual spots.*

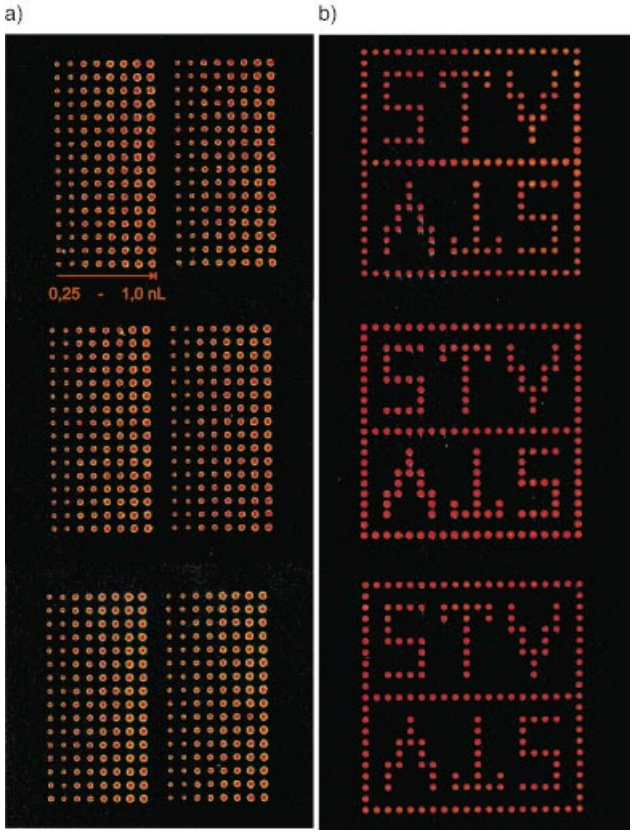


Figure 5. *Demonstration of the long-range homogeneity of the dendrimer-modified glass cover slides. a) Array of DNA oligomers covalently attached to a dendrimer-modified surface* ***5c****. Volumes of the amino-modified DNA oligomer ranging from 0.25 – 1.0 nL were deposited on the cover slide to form six identical patterns. The array was used for hybridization experiments with the complementary 5′-Cy5-labeled target oligomer, applied as a 1 nmol L^{-1} solution. Signals were measured with a fluorescence scanner (pmt500). b) Array of biotin-binding protein streptavidin covalently attached to a dendrimer-modified surface* ***5b****. 1 nL of a 20 μmol L^{-1} streptavidin solution was deposited in each spot. The array was used for capturing a twofold modified, double-stranded DNA fragment containing a biotin substituent and a Cy5-fluorophore at its two 5′-ends. After binding of the DNA target through a streptavidin – biotin interaction, the fluorescence signals were detected by fluorescence analysis (pmt500).*

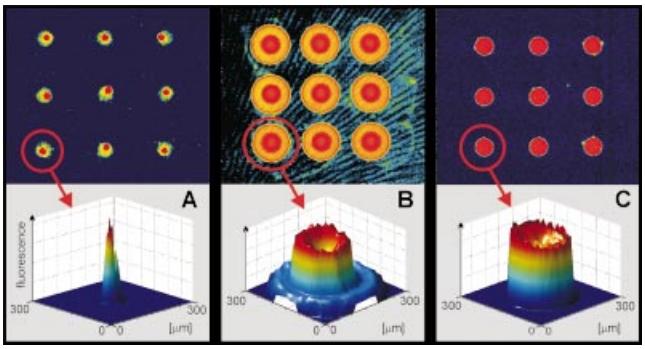


Figure 6. *Comparision of the spot homogeneity of various surfaces: a) an epoxysilane-activated slide, b) an array printed on a nitrocellulose-coated slide, and c) a PAMAM dendrimer slide. All three slides were spotted with 500 pL of a 10 µmol L^{-1} solution of amino-capture oligomer. Hybridization was carried out with a 1 nmol L^{-1} solution of a 5′-Cy5-labeled, complementary target oligomer. As indicated by the three-dimensional representation of the signal distribution (pmt500), the dendrimer slide reveals sharp and highly homogeneous spots.*

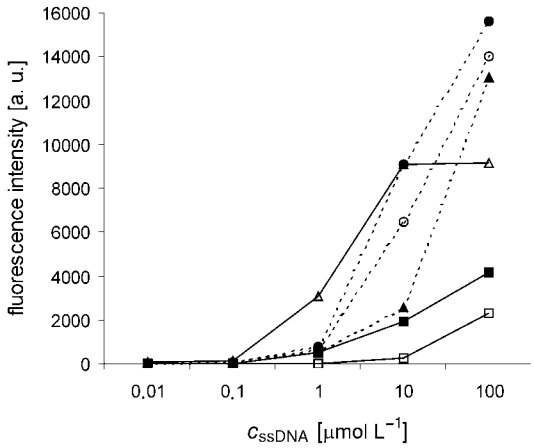


Figure 7. *Investigation of the binding capacity of the various activated glass surfaces. 250 pL of a single-stranded DNA oligomer (ssDNA) containing a 3′-amino group and a 5′-Cy5 fluorophore were deposited on the activated supports* ***3*** *(■),* ***4****(△),* ***5a*** *(▲),* ***5b*** *(●), and* ***5c*** *(○) in concentrations of 0.01 µmol L^{-1} – 100 µmol L^{-1}. For comparison, a commercially available epoxysilane-coated slide (□) was also studied. The signals were obtained with a fluorescence scanner (see Figure 5), and the average of the pixel intensities within a spot was calculated.*

available activated slides are compared with a dendrimer slide. Hybridization was performed with a solution of a 5′-Cy5-labeled oligomer (1 nmol L^{-1}) using either an array spotted on an epoxysilane-activated slide (Figure 6a), a nitrocellulose covered slide (Figure 6b), or a PAMAM dendrimer slide (Figure 6c). As indicated from the three-dimensional representation of the signal intensities, the dendrimer activation led to sharp spots with the best homogeneity of the signal distribution. This result is particularly important since the homogeneity within an oligomer spot is crucial for quantitative analysis using commercially available software tools.[13]

Immobilization efficiency: The binding capacity of the activated supports is most important for the high density coupling of DNA oligomers, which allows high signal-to-background ratios, and thus, enables lowest limits of detection upon microarray hybridization analysis. To systematically study the binding capacity of surfaces **3**, **4**, and **5a**–**c**, a doubly functionalized oligonucleotide containing an amino group at its 3′-end and a Cy5 fluorophore at its 5′-end was used. For the preparation of DNA oligomer arrays, a solution of this oligomer (250 pL) was dropped onto the activated glass supports. The concentration of the oligomer solution was varied between 0.01 µmol L^{-1} and 100 µmol L^{-1}. For comparison, additional arrays were prepared by using commercially available, epoxy-silylated slides, which thus allowed the direct coupling of the amino-modified oligomer without any additional linker system. After spotting, the chemical coupling was allowed to proceed for two hours in a humidity chamber; unbound oligomers were removed by washing, and the fluorescence intensity was determined with a fluorescence scanner. As shown in Figure 7, the epoxysilane-coated slides exhibited the lowest binding capacity, possibly as a result of the the missing linker system. Similarly, only small amounts of oligomers were attached on surface **3**. The isothiocyanate-modified surface **4** revealed higher signal intensities, especially in the case of the depostion of low concentrated oligomer solutions. However, saturation of signal intensity was reached with surface **4** at an oligomer concentration of 10 µmol L^{-1}, and no further increase of signal intensity was obtainable even at high oligomer concentrations deposited. In contrast, no such saturation was observed with the dendrimer surfaces **5a**–**c**, most likely as a consequence of the enhanced amount of binding sites provided by the three-dimensional linker system. The comparison of surfaces **5a**–**c** revealed that the immobilization efficiencies increase with the PDITC content used during the generation of the linker system.

To determine the absolute amount of ssDNA attached, additional autoradiography experiments have been carried out. According to the results of these experiments, the surface loading of **5b** is approximately 150 fmol mm^{-2}, when immobilization was performed with a solution containing 10 µmol L^{-1} of the 5′-amino-oligomer. This coverage is equivalent to approximately one molecule per 10 nm^2. In contrast, lower oligomer densities of about 70 fmol mm^{-2} were determinated for surface **4**. These results suggest that no significant increase in signal intensity would be obtained if ssDNA was deposited on the dendritic surfaces **5a**–**c** at concentrations higher than 100 µmol L^{-1}. Loadings of less than 5 fmol mm^{-2} were measured for the commercially available epoxysilane-modified slides. Thus, experiments with both radioactively and fluorophore-labeled ssDNA oligomers indicated that an approximatly twofold higher loading is achieved with the dendritic surfaces, despite the 64 amino groups present in each of the dendrimer monomers. This suggests that many functional groups of the PAMAM dendrimer are consumed to form the polymeric network (Scheme 1b). To enhance the immobilization capacity, however, the number of active binding sites of dendrimer-modified surfaces can be increased by employing monofunctional or heterobifunctional linker reagents, such as glutaric anhydride or *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester during the last activation step, instead of using the homobifunctional linkers PDITC or DSG. Compared to the linear linker surfaces **3** and **4** an approximately

eightfold higher loading was observed in hybridization experiments with such noncrosslinked dendritic surfaces (data not shown).[14]

Regeneration stability: A major motivation to develop the dendrimer surfaces was their applicability as a support for the preparation of reusable DNA microarrays. To test the physicochemical stability of the linker system, we compared the regeneration stability of the dendrimer surface **5b** with that of the conventional surface **4** (Figure 8). For this, two different regeneration procedures were applied: After hybridization of the

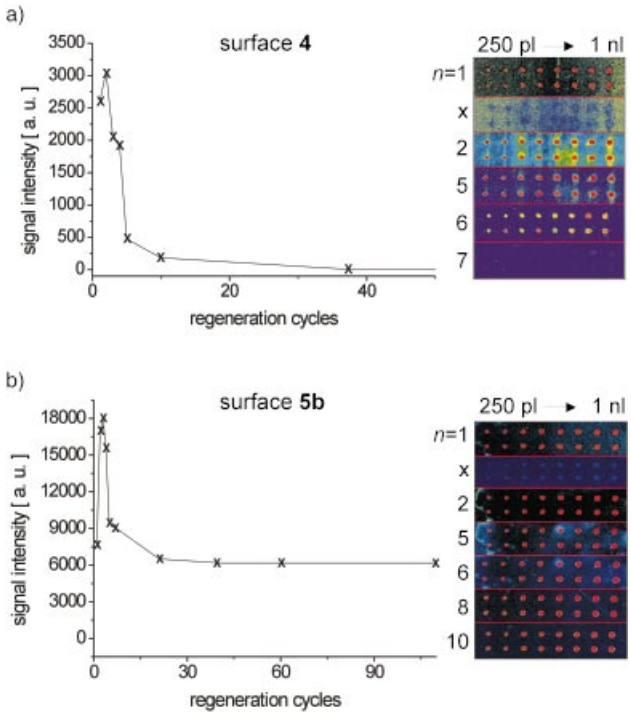


Figure 8. *Comparison of the stability of amino-modified surfaces* ***4*** *(a) and* ***5b*** *(b), containing a linear or a dendrimer-based linker system, respectively. The microarrays were prepared by deposition of 0.25 – 1.0 nL of a 5′-amino-modified capture oligomer (10 μmol L^{-1}) on the activated surfaces. The arrays were used for hybridization detection of a complementary 5′-Cy5-labeled target oligomer applied as a 1 nmol L^{-1} solution. The signals were determinated with a fluorescence scanner. Alkaline-stripping conditions were used for regeneration of the arrays. Each regeneration cycle corresponds to one hour incubation in an alkaline-stripping solution (see text for details). Fluorescence images of identical regions of the arrays are shown in each case on the right. The images were obtained after regeneration and rehybridization steps, as indicated by the value of* n. *Image* x *was obtained after the first regeneration.*

microarrays with 1 nmol L^{-1} of the Cy5-labeled target oligomer, the arrays were regenerated by using either an alkaline solution containing NaOH (50 mmol L^{-1}) and SDS (0.1 %, w/v) at room temperature ("alkaline stripping"), or by using a neutral pH 7.5 phosphate buffer containing SDS (0.1 %, w/v) at 95 °C ("thermal stripping"). To ensure that the removal of the Cy5-labeled target oligomer is greater than 99 %, the arrays have to be incubated for about one hour in the alkaline medium, whereas a treatment of about two minutes was sufficient when slides are regenerated by thermal stripping. Many cycles of regeneration were carried out on the surfaces **4** and **5b**; however, the time-consuming rehybridization of the stripped arrays was skipped after most of the regeneration steps. Instead, the arrays were kept in the regeneration medium for prolonged periods of time. As indicated in Figure 8a, surface **4** was not particularly resistant against the regeneration conditions. The signal intensities of the rehybridized arrays decreased significantly with each regeneration cycle, and after five cycles only a very low signal intensity was detected. Similar results were obtained with surface **3** (data not shown). Thus, in our hands, the silylated surfaces with conventional linker systems appeared to be inappropriate to serve as support for the preparation of reusable microarrays.

In contrast, the dendrimer-modified surfaces, in particular **5b**, revealed a remarkable stablility even after numerous cycles of regeneration. Interestingly, microarrays based on surface **5b** showed an increased signal intensity after one cycle of alkaline stripping and rehybridiziation (Figure 8b). In subsequent hybridization experiments the signal intensities decreased slightly to reach a plateau, which remained constant for more than 100 simulated regeneration cycles. The tenth rehybridization, carried out after treating the array with alkaline stripping conditions corresponding to 110 regeneration cycles, still led to signal intensities of about 80 % of the initial hybridization signals and of about 35 % compared with the maximum signal intensity observed after the third hybridization. Moreover, significantly decreased background signals were evident after several cycles of regeneration, indicating a reduced nonspecific binding of the Cy5-labled target oligomers. Thus, the use of regenerated arrays led to elevated signal-to-noise (*S*/*N*) ratios. The *S*/*N* ratio after the first hybridization was about 100:1, after 20 regeneration cycles it increased to approximatly 500:1, and after more than 100 regeneration cycles *S*/*N* ratios greater than 5000:1 were observed. This indicated that alkaline-stripped dendrimer arrays allow a significantly higher sensitivity compared to those used for the initial hybridization experiments. Under the thermal stripping conditions, the stability of the surfaces was reduced. In that case, a continuous decrease in signal intensity was observed. The tenth rehybrization after 110 simulated regeneration cycles led to remaining signal intensities of about 40 % compared to those obtained from the initial hybridization.

Protein immobilization: Initial experiments were carried out to test the versatility of the dendrimer-modified surfaces for immobilizing amino-containing compounds other than DNA oligomers. For this, an aqueous solution containing the biotin-binding protein streptavidin was spotted onto the activated surface **5b**. After the removal of excess material by washing, the immobilization of the protein was tested by capturing a biotin-derivatized double-stranded DNA fragment which contained an additional Cy5 label attached to one of its 5′-ends. A fluorescence image of the protein array is shown in Figure 5b, which indicates the highly homogeneous spot pattern throughout the entire surface of the glass slide. This result again emphasizes the high quality and the versatility of the novel dendrimer surfaces developed.

Conclusions

DNA and protein microarray technology essentially require chemically activated surfaces which allow the highly reproducible and homogeneous coupling of bioorganic compounds. Since the use of glass microscope slides is most common, we have developed an optimized functionalization procedure for silica surfaces based on 1) a highly homogeneous aminosilylation of the glass support and 2) a subsequent in situ generation of a polymeric thin-film layer comprising a dendritic monomer. The resulting surfaces not only have a very high binding capacity for amino-modified compounds, but also reveal an outstanding resistance against the harsh washing and regeneration conditions, necessary to recover DNA microarrays after hybridization analyses. As an additional advantage, the surface properties can be modulated by the use of various crosslinking agents, thereby allowing, for instance, the generation of activated supports of variable hydrophobicity. The versatility of the dendrimer-modified surfaces was demonstrated by their employment in the production of both DNA and protein microarrays. We anticipate that the novel dendrimer surfaces will allow the production of dense arrays comprising nucleic acids, proteins, and low-molecular-weight compounds.

Such arrays are useful for applications ranging from genomics and proteomics to the various fields of biomedical diagnostics and high-throughput screening. Further studies are currently in progress on the application of dendrimer surfaces as a platform for the detection of protein – ligand interactions, such as the immunosorptive binding of antigens through surface-bound antibodies, or the analysis of single-nucleotide polymorphisms.[15]

Experimental Section

DNA oligomers and proteins: Streptavidin was purchased from IBA (Göttingen) and DNA oligomers were from Interactiva (Ulm). 5′-Amino-modified DNA oligomer used for attachment to the activated surfaces: 5′-NH_2-AGC GGA TAA CAA TTT CAC ACA GGA-3′. 5′-Cy5-Labeled DNA oligomer used for hybridizations: 5′-Cy5-TCC TGT GTG AAA TTG TTA TCC GCT-3′. Twofold modified oligomers for investigation of immobilization efficiencies: 1) for detection by fluorescence: 5′-Cy5-AGC GGA TAA CAA TTT CAC ACA GGA-NH_2-3′ and 2) for detection by autoradiography: 5′-^{32}P-AGC GGA TAA CAA TTT CAC ACA GGA-NH2-3′.

Instrumentation: Laser scanning system: GenePix4000 (Axon); piezo-driven spotting device: Robodrop (BIAS, Bremen); UV/Vis Spectrophotometer: Lamda 2 (Perkin Elmer); scintillation analyzer: liquid scintillation analyzer 2500 TR (Packard).

Synthesis of disuccinimidylglutarate (DSG): DSG was prepared by dissolving glutaric acid (17 mmol), *N*-hydroxysuccinimide (35 mmol), and *N*,*N*′-dicyclohexylcarbodiimide (35 mmol) under dry conditions in amine-free DMF (50 mL). The solution was stirred for 24 h and then filtered under vacuum to remove the precipitated urea derivative. The solvent was removed on a rotary evaporator at 60 °C under reduced pressure (30 mbar). The crude product was dissolved under reflux in CH_2Cl_2 and reprecipitated by adding hexane. If necessary, an additional flash-chromatography purification (silica gel; CH_2Cl_2/$CH_3COOC_2H_5$, 1:1) can be carried out to remove side products remaining. The yields of isolated products were 60 %. MS (FAB+, NBA): *m/z* (%): 327 (28) [$M+H^+$], 212 (61), 154 (100), 136 (93); ^{1}H NMR (360 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 1.6$ (t, ^3J(H,H) = 7 Hz, 4 H; CH_2), 2.2 (q, ^3J(H,H) = 8 Hz, 2 H, CH_2), 2.8 (m, 8 H, CH_2); IR (KBr): $\tilde{\nu}$ [cm^{-1}] = 2963, 2852 (C–H); 1820, 1784, 1732 (C=O); 1262, 1205, 1077, 869, 804.

Silylation procedures: All cleaning and modification steps were carried out in a glass tube under heavy stirring. The microscope glass slides (Menzel Gläser, Braunschweig) were first degreased by ultrasonic treatment in CH_2Cl_2, and subsequently cleaned in freshly prepared, hot piranha solution (H_2SO_4/H_2O_2, 2:1) for 30 min. Thereafter, the slides were rinsed in bidistilled water and dried for 10 min at 80 °C in a nitrogen atmosphere. The initial modification of glass slides **1** to obtain surface **2** (Scheme 1 a) was achieved by treatment with 3-aminopropyltriethoxysilane (APTS, Sigma-Aldrich) under various conditions: For silylation under water-free conditions the slides were stirred in a mixture of toluene/APTS/diisopropylethylamine (89:10:1 v/v) at 80 °C in a nitrogen atmosphere for between one and twenty hours. The amino-silylated slides were washed thoroughly with toluene and acetone. The similar method was used for silylation in the presence of water by adding 100 ppm of deionized water to the above toluene/APTS/diisopropylethylamine solution. To produce surfaces **2** by silylation in a ethanol/water solution, the cleaned slides were treated with a mixture of ethanol/H_2O/APTS (95:3:2 v/v) for one up to twenty hours, and then the slides were cleaned with ethanol and acetone as described above. All silylated slides were finally dried for 10 min at 110 °C prior to the further activation.

Quantitative determination of amino functionalization using rhodamine-B: To quantify the amount of amino groups present after amino-silylation, sulfonyl chloride activated rhodamine B (Molecular Probes) was covalently coupled with the amino groups of surface **2** through a sulfonamide bond. For this, the slides were incubated for 1 h in a solution containing the rhodamine B derivative (10 μmol L^{-1}) and pyridine (1 % v/v) in CH_2Cl_2 under water-free conditions. After the samples had been washed with CH_2Cl_2 and ethanol, the absorbance at 550 nm was determined using a UV/Vis spectrophotometer. A similar protocol, using tetramethylrhodamine cadaverine (Molecular Probes) instead of the rhodamine B sulfonyl chloride was employed to determine the quality of activated surfaces **3** through **5 c**.

Synthesis of linear linker surfaces 3 and 4: Slides activated with conventional linear linkers were synthesized by incubation of amino-silylated slides **2** for 2 h in a solution containing either disuccinimidylglutarate (DSG, 10 mmol L^{-1}) in CH_2Cl_2 supplemented with diisopropylethylamine (1 %, v/v) to yield surface **3**, or in a solution containing 1,4-phenylenediisothiocyanate (PDITC, 10 mmol L^{-1}; Fluka, Neu-Ulm) in CH_2Cl_2, supplemented with pyridine (1 %, v/v) to yield surface **4**. The slides were washed three times in CH_2Cl_2 and dried in a nitrogen stream. The activated slides were stored in an argon atmosphere at −20 °C.

In situ generation of the polymeric linker systems: In a first step, the dendritic PAMAM starburst monomer (100 μL of a 10 % (w/v) solution; Sigma-Aldrich) was deposited on a slide containing the activated surfaces **3** or **4**, and the slide was incubated overnight. Excessive monomer was removed by washing with ethanol and acetone and then the slides were dried in a nitrogen stream. The PAMAM monomers attached were cross-linked and activated for two hours with either a solution of DSG in CH_2Cl_2 (10 mmol L^{-1}) containing diisopropylethylamine (1 %, v/v), or with a solution of PDITC in CH_2Cl_2 (10 mmol L^{-1}) containing pyridine (1 %, v/v) under an inert atmosphere. The slides were thoroughly rinsed with CH_2Cl_2 and

acetone, and immediately used for the coupling with amino-modified oligonucleotides as described below.

Covalent attachment of oligonucleotides and proteins: For the attachment of oligonucleotides, typically 0.25 – 1.0 nL of a solution of the 5′-amino-modified oligonucleotide in water (10 μmol L^{-1}) was droped onto the activated slides using a piezo-driven spotting device, and the slides were incubated overnight in a chamber under saturated humidity. Subsequently, the slides were air-dried and submerged in a solution containing 6-aminohexanol (150 mmol L^{-1}) and *N*,*N*′-diisopropylethylamine (50 mmol L^{-1}) in DMF to quench the remaining coupling groups. After washing with DMF, acetone, and water, the slides were used for hybridization, or were stored at −20 °C. A similar protocol was used for the preparation of protein microarrays. Briefly, the protein was dissolved in water (10 – 20 μmol L^{-1}), spotted onto the activated support, and then the slides were incubated overnight at 4 °C. Quenching of remaining binding sites was carried out in water instead of DMF.

Hybridization experiments: Nucleic acid hybridization experiments were carried out in polypropylene vessels. To reduce nonspecific binding of the 5′-Cy5-labeled oligonucleotides, the microarrays were pretreated for one hour with a solution containing herring sperm DNA (100 μg mL^{-1}; Boehringer Mannheim) in TBS buffer (20 $mmol^{-1}$ tris-Na-citrate, 150 $mmol^{-1}$ NaCl, pH 7.35). Subsequently, the microarray was transferred to a hybridization chamber and covered with the hybridization solution, containing the 5′-Cy5-labeled complementary target oligonucleotide (1 nmol L^{-1}) in TETBS (TBS, 5 mmol L^{-1} EDTA, 0.05% v/v Tween20), supplemented with herring sperm DNA (100 μg mL^{-1}). Hybridization was carried out at 25 °C for one hour. The chip was then washed twice for 10 min with 2 × SSC (0.3 $mmol^{-1}$ NaCl, 30 $mmol^{-1}$ tris-Na-citrate)/0.1% SDS (sodium dodecylsulfate) followed by 0.2 × SSC/0.1% SDS twice. The fluorescence intensity was measured by using a microarray laser scanning system.

Investigation of loading capacities by autoradiography: To quantify the amount of capture oligomers attached to the glass slides, a 5′-^{32}P-labeled and 3′-NH_2-modified DNA oligomer was used. 100 ppm of this ^{32}P-labeled oligomer were added to a solution containing the identical unlabeled oligomer in water (10 μmol L^{-1}). Activated slides were incubated with the oligomer solution and washed as described above. For quantitative determination the slides were submerged in a scintillation cocktail (LSC Ultima Gold XR, Packard), and radioactive decays were counted with a scintillation analyzer.

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