Journal of Fluorescence, Vol. 15, No. 2, March 2005 (© 2005)
DOI: 10.1007/s10895-005-2524-4

Optimization of Gold Nanoparticle-Based DNA Detection for Microarrays

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Received June 1, 2004; accepted December 30, 2004

DNA microarrays are promising tools for fast and highly parallel DNA detection by means of fluorescence or gold nanoparticle labeling. However, substrate modification with silanes (as a prerequisite for capture DNA binding) often leads to inhomogeneous surfaces and/or nonspecific binding of the labeled DNA. We examined both different substrate cleaning and activating protocols and also different blocking strategies for optimizing the procedures, especially those for nanoparticle labeling. Contact angle measurements as well as fluorescence microscopy, atomic force microscopy (AFM), and a flatbed scanner were used to analyze the multiple-step process. Although the examined different cleaning and activating protocols resulted in considerably different contact angles, meaning different substrate wettability, silanization led to similar hydrophobic surfaces which could be revealed as smooth surfaces of about 2–4 nm roughness. The two examined silanes (3-glycidoxypropyltrimethoxysilane (GOPS) and 3-aminopropyltriethoxysilane (APTES)) differed in their DNA binding homogeneity, maximum signal intensities, and sensitivity. Nonspecific gold binding on APTES/PDC surfaces could be blocked by treatment in 3% bovine serum albumin (BSA).

KEY WORDS: DNA microarray; silanes, fluorescence; gold nanoparticle; BSA blocking; AFM.

INTRODUCTION

DNA detection methods are of growing interest for diagnostics, risk assessment, and other applications. For a highly parallel read out, DNA microarrays are of special interest [1,2]. Therefore, usually glass or silicon substrates are functionalized for DNA binding. Immobilization of different DNA capture sequences enables the examination of many different aspects at the same time. After hybridization with sample molecules, specific binding of complementary DNA probe can be detected by tagged molecules, using radioactivity, fluorescent dyes, or metal nanoparticles [3,4]. Although fluorescence labeling is a commonly used and well-established method, it has also some disadvantages, like a low stability of fluorescent dyes and the requirement for expensive read out systems. For a parallel read out of gold nanoparticles, optical and electrical detection methods were described [5–8]. Silver enhancement of gold nanoparticles allows an optical read out by simple flatbed scanners [6,9].

The quality of solid-phase-based arrays depends on the immobilization efficiency of capture probes. To ensure a reproducible and quantitative signal, a controlled DNA immobilization procedure is required. There are two well-known approaches to microarray manufacturing: on the one hand, in situ methods comprising the on-chip synthesis of capture probes, on the other hand, ex situ synthesizing of the capture molecules and their following transfer to the array substrate. This postsynthetic approach can be performed by electrostatic or covalent binding. Therefore, different DNA attachment strategies are possible as described in the literature by Zammateo *et al.* [10]. But

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often, inhomogeneous surfaces lead to less reproducible signals, and nonspecific binding hampers the significance of the results obtained.

For that reason, in preparing this paper, different cleaning strategies as a prerequisite for substrate functionalization were examined, characterizing two different surface modification methods with silanes (3-glycidoxypropyltrimethoxysilane (GOPS); 3-aminopropyltriethoxysilane (APTES)) so as to provide functional groups for covalent attachment of amino-modified DNA. With its epoxy-groups, GOPS enables direct binding of the NH_2-oligonucleotides, whereas for the amino–silane APTES, like 1,4-phenylenediisothiocyanate (PDC) is necessary to connect the amino-groups of APTES and the amino-modified oligonucleotides. The immobilized DNA spots were hybridized with fluorescence and gold nanoparticle-labeled oligonucleotides in order to characterize their homogeneity. The hybridized fluorescent samples were detected by a fluorescence microscope and/or a microarray reader. For reducing nonspecific binding of gold-tagged DNA molecules, we examined different blocking strategies with 6-mercapto-1-hexanol, ethanolamine, and bovine serum albumin (BSA). After silver enhancement, the hybridized gold nanoparticles were optically read out by a flatbed scanner, and individual particles were visualized by AFM.

MATERIALS AND METHODS

Preparation of DNA-Nanoparticle Complexes

The 5′-alkylthiolated oligonucleotides (SH-C_6-CATAGAATCAAGGAGCACATGCTGAAAAAA; JenaBioscience, Jena, Germany) were desalted and separated from dithiothreitol (DTT) by column-chromatography with NAP-10 (Ammersham Pharmacia Biotec, Freiburg, Germany). The cleaned oligonucleotides were incubated with colloidal gold solution (Plano, Wetzlar, Germany) using a modified protocol from the literature [11,12] leading to covalent binding of active thiol-groups on the surface of the 30-nm-sized gold particles. The concentration of the DNA-nanoparticle complexes was determined by UV–Vis spectroscopy at 525 nm and final concentration was set to 1 OD in 0.1 M NaCl/0.01 M sodium phosphate buffer. The probes were stored 1:1 in glycerol (v/v) at -20°C [13] and washed twice with 0.1 M NaCl/0.01 M sodium phosphate buffer directly before use.

Substrate Modification

To enable the binding of amino-modified oligonucleotides, the surface was covered with either epoxy groups by GOPS or amino groups by APTES (Sigma-Aldrich, Taufkirchen, Germany). NH_2-oligonucleotides are able to bind directly to epoxy groups on the GOPS surface. The indirect binding to the surface-bound amino groups from APTES was realized by the bifunctional crosslinker PDC. Both reaction schemes are shown in Fig. 1. For the substrate preparation, different cleaning and activating strategies were examined using modified protocols from the literature (Table I). In all further experiments, the glass slides were precleaned for 10 min each in acetone, ethanol, and deionized water in an ultrasonic bath; and thereafter activated for 10 min in 1:1:1 hydrochloric acid, hydrogenperoxide, and deionized water. After drying at 120°C for 10 min the silanization was either performed for 6–8 hr at 70°C in 10 mM GOPS in dry toluene or in 1% APTES in acetone at 37°C for 1 hr (for fluorescence labeling) or 2 min in 1% APTES/95% acetone/dH_2O at room temperature (for nanoparticle labeling) according to modified protocols from the literature [14,15], respectively. After rinsing the GOPS-silanized probes in toluene, ethanol, and deionized water, the substrates were dried under a nitrogen flow and ready for further processing. The APTES-silanized probes were washed in acetone, dried for 45 min at 105°C, treated for 2 hr with a solution of 0.2% PDC in 10% pyridine/dimethylformamide (Sigma-Aldrich, Taufkirchen, Germany), washed with methanol and acetone: and dried in a stream of nitrogen.

DNA Immobilization

The 5′-aminomodified oligonucleotides (JenaBioscience, Jena, Germany) with a complementary sequence (NH_2-C_6-TTTTTTCAGCATGTGCTCCTTGATTCTATG), a sequence containing one (NH_2-C_6-TTTTTTTCAGCATG**G**GCTCCTTGATTCTATG) or three mismatches (NH_2-C_6-TTTTTTCAGCAT**TAT**CTCCTTGATTCTATG): and a totally noncomplementary sequence (NH_2-C_6-ACTGACTGACTGACTGACTGACTGGGCGGCGACCT or NH_2-C_6-TAAGGTTCATGAGCCTTTCGAGGAGATGAAGTGTATTGGG) were diluted in 0.1 M KOH pH 8.5 for GOPS surfaces [14] and in 0.1 M $NaHCO_3$ pH 8.5 for APTES-functionalized substrates [15]. Different concentrations of the oligonucleotide solutions (0.5–100 μM) were manually spotted on the silanized surfaces (1 μL per spot) and incubated overnight at 37°C in a humidity chamber. After DNA immobilization, the substrates were rinsed in different washing solutions: 0.1% Triton X-100, hydrochloric acid pH 4, 0.1-M potassium chloride

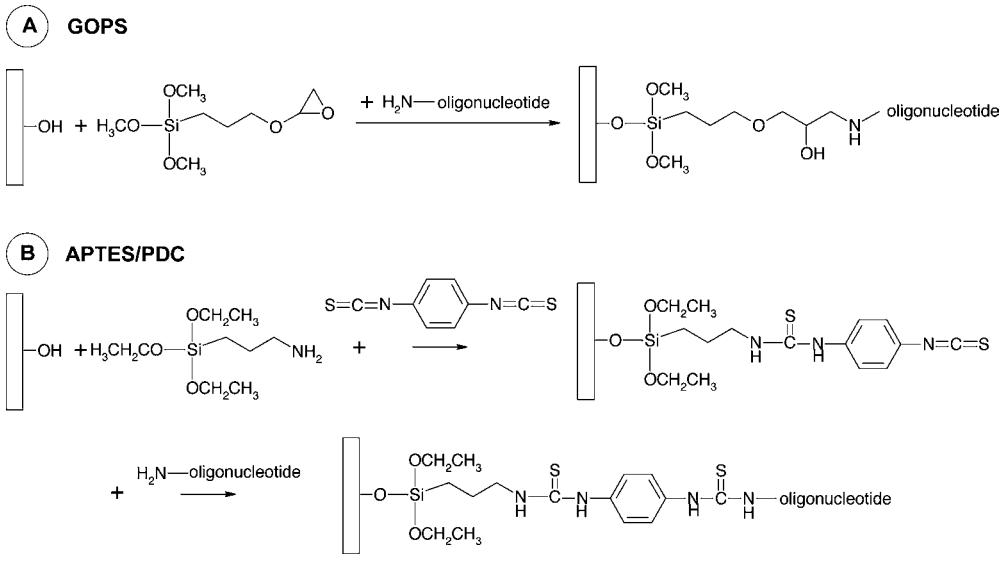

Fig. 1. Surface modification with silanes for DNA attachment. (A) Direct binding of amino-modified DNA by epoxy-groups of 3-glycidoxypropyltrimethoxysilane (GOPS). (B) Amino attachment chemistry of 3-aminopropyltriethoxysilane (APTES). The binding of amino-modified oligonucleotides works indirectly by the bifunctional crosslinker 1,4-phenylenedisiothiocyanate (PDC).

and deionized water. For direct characterization of the binding morphology we used the complementary sequence, labeled with a 3′-fluorescein isothiocyanate (FITC).

Covalent Versus Nonspecific DNA Binding

To elucidate the kind of DNA attachment to the amino-silane layer, we incubated the APTES/

Table I. Comparison of Different Cleaning and Activating Protocols from the Literature Regarding Contact Angles of Cleaned and Silanized Glass Substrates

Literature	Applied Protocol	Contact angles after		
		Cleaning	GOPS	APTES/PDC
[24]	30 min in 1:1 methanol:HCl conc.; 30 min in H_2SO_4 conc.	10.3 ± 0.6	59.0 ± 3.2	—
[25]	30 min in 1:1 methanol:HCl conc.; 30 min. in H_2SO_4 conc.; 10 min in boiled deionized water	19.2 ± 9.8	58.7 ± 3.1	—
[26]	15 min in Piranha solution (3:1 H_2SO_4 conc.:H_2O_2)	30.5 ± 8.9	54.7 ± 2.1	—
[27]	30 min in Piranha solution (3:1 H_2SO_4 conc.:H_2O_2)	7.2 ± 0.6	59.3 ± 2.7	—
Modified after [26]	10 min per wash in acetone, ethanol, and dH_2O in an ultrasonic bath; 15 min in Piranha solution (3:1 H_2SO_4 conc.:H_2O_2)	23.3 ± 8.6	57.3 ± 3.9	—
Modified after [27]	10 min per wash in acetone, ethanol, and dH_2O in an ultrasonic bath; 30 min in Piranha solution (3:1 H_2SO_4 conc.:H_2O_2)	9.0 ± 1.4	54.1 ± 2.9	—
[18]	10 min per wash in acetone, ethanol, and dH_2O in an ultrasonic bath; 10 min in 1:1:1 HCl conc.:H_2O_2:dH_2O	21.2 ± 3.5	60.9 ± 1.4	53.8 ± 3.0

PDC-modified substrates with ethanolamine (100 mM, 30 min) before adding the oligonucleotides in order to block possible emerging of binding groups. In a second approach, the DNA was applied on pure APTES-silanized surfaces without addition of the cross-linking reagent PDC.

Blocking

To reduce nonspecific binding of target oligonucleotides to the silane layer, the glass substrates were blocked after the capture DNA immobilization for 15 min in 50 mM ethanolamine + 0.1% sodium dodecyl sulfate (SDS) in Tris pH 9 (GOPS surfaces) and for 5 min in 0.1% ammonium hydroxide solution (APTES surfaces), respectively. For blocking any surface still available to nonspecific gold binding, the substrates were immersed for 1 hr in 10-mM 6-mercapto-1-hexanol (Sigma-Aldrich, Taufkirchen, Germany) and/or for 2 hr in a phosphate buffered saline (PBS) solution, pH 7.4, containing different concentrations of BSA up to 3%, using a modified protocol from Wirtz *et al*. [16]. The passivated substrates were washed in PBS pH 7.4 + 0.05% Tween 20, PBS pH 7.4, and finally deionized water for 5 min each.

Hybridization

The fluorescence-labeled target (FITC-CATAGAATCAAGGAGCACATGCTG) was diluted in 5× sodium chloride/sodium citrate (SSC) + 0.1% SDS to a concentration ranging from 0.002 to 2 μM. The nanoparticle-labeled DNA was diluted in 0.1 M NaCl/0.01 M sodium phosphate buffer to 1 OD, determined by UV–Vis spectroscopy at 260 and 525 nm, respectively. The substrates were incubated at 40°C for 3 hr in a humidity chamber, then rinsed in washing solutions of different SSC contents after standard hybridization protocols (for fluorescence) or in 0.1 M NaCl/0.01 M sodium phosphate buffer (for gold nanoparticles): and dried in a stream of nitrogen.

Silver Enhancement

The gold-labeled probes were incubated for 10 min in a self-made silver acetate/hydroquinone solution (Sigma-Aldrich, Taufkirchen, Germany) using a modified protocol [9], and shortly rinsed in deionized water and dried under a nitrogen flow afterwards [17].

Contact Angle Measurements

The surface hydrophobicity of the substrates was studied with a Contact Angle System OCA 20 (data-physics instruments, Filderstadt, Germany) at room temperature using water as liquid. The measured contact angles were mean values from at least three measurements. Left and right contact angles usually varied by less than 5% and were therefore averaged.

Imaging

The fluorescence-labeled probes were optically characterized by both a fluorescence microscope (Zeiss Axiotech; Zeiss, Jena, Germany) and a microarray reader (MicroArrayReader V 1.20; Jena-Optronik, Jena, Germany). The generated 8-bit grayscale images were analyzed with image manipulation programs. From the given data the background level has been subtracted. The silver-enhanced gold nanoparticles were optically detected with a flatbed scanner (AGFA Duoscan T2500, Agfa Deutschland, Köln, Germany), and individual particles were visualized by atomic force microscopy (AFM) (Nano Scope III, Dimension 3100; Digital Instruments, Santa Barbara, CA) using the tapping mode in air. The scan size was 5 × 5 or 10 × 10 μm, respectively.

RESULTS

The success of solid-phase microarrays depends on the efficiency of immobilized capture probes. Therefore, the covalent attachment of presynthesized oligonucleotides requires a sufficient and reproducible DNA immobilization. Here, we examined two silanization methods for enabling the covalent binding of amino-modified oligonucleotides on glass substrates.

Cleaning and Activating Methods

For optimizing the silanization procedures, the surfaces were cleaned to remove any contamination and thereafter activated to increase the density of reacting OH-groups. The result was characterized by contact angle measurements and AFM imaging. Although all cleaning procedures generated hydrophilic surfaces and therefore small contact angles, the measured values differed considerably from 7.2 to 30.5° (Table I). The smallest contact angles (7.2 ± 0.6°) could be obtained after treatment in Piranha solution (3:1 sulfuric acid:hydrogenperoxide) for 30 min. An additional precleaning with acetone, ethanol, and water had no significant effect on these values. However, the significant differences between the various cleaning procedures regarding the contact angles disappeared after the silanization procedures. The GOPS-silanized

substrates showed strongly hydrophobic surfaces observable in the increased contact angles of $60.9 \pm 1.4^{\circ}$ for precleaning in acetone/ethanol and activation in hydrochloric acid/hydrogenperoxide [18]. Therefore, we used this protocol for all further experiments. Also, silanization with APTES/PDC led to increased contact angles of $53.8 \pm 3.0^{\circ}$ pointing to hydrophobic surface properties and a successful surface modification. Furthermore, the cleaned substrates were characterized by AFM imaging (Fig. 2) revealing clean and smooth surfaces with a surface roughness below 1 nm. The GOPS silanization led to slightly rougher surfaces with about 4 nm in height corrugation. For APTES/PDC substrates, the optimal surface roughness of about 2 nm was obtained by silanization in 1% APTES in 95% acetone/dH_2O for 2 min at room temperature.

Effect of the DNA Attachment Chemistry on the Signal Intensities and Morphology

The aim of this paper is the optimization of nanoparticle-based DNA detection by using gold-labeled oligonucleotides as target DNA. To characterize the binding properties of different silanization procedures, first fluorescence-labeled target DNA was utilized because of its well-established hybridization and detection methods. Amino-modified capture oligonucleotides were attached to the binding groups of the silanized glass substrates, using different concentrations of complementary DNA (0.5–100 μM) to estimate the binding capacity of both silane surfaces. Furthermore, a noncomplementary capture sequence was also immobilized to investigate nonspecific binding. The bound oligonucleotides were hybridized with fluorescent target oligonucleotides in different concentrations (0.002–2 μM) to evaluate the hybridization signal sensitivity on the silanized surfaces. The substrates were scanned in a microarray reader and the received fluorescence signals were analyzed with image manipulation programs yielding relative brightness values (Fig. 3). The highest brightness values could be detected on GOPS surfaces (71.7 ± 4.4 AU for 100 μM capture oligonucleotides and 0.2-μM FITC-labeled target DNA, Fig. 3A). Unexpectedly, the same capture concentration hybridized with a higher concentration of FITC-labeled DNA (2 μM) exhibited with 51.4 ± 11.1 AU smaller signal intensities. On APTES-coated surfaces, the maximum brightness values were lower as on GOPS-coated surfaces but did not significantly differ with 49.9 ± 6.5 AU vs. 47.4 ± 9.1 AU for 100 μM capture oligonucleotides and 0.2 or 2 μM FITC-labeled target DNA, respectively (Fig. 3B). Although GOPS-surfaces yielded higher maximum fluorescence signals, on APTES-silanized substrates the capture oligonucleotides could be detected also for lower concentrations. So on APTES, 5 μM capture DNA gave signals even with 0.02 μM FITC-labeled DNA whereas on GOPS this capture DNA amount was only detectable with much higher concentrations of fluorescent oligonucleotides.

A closer look at the DNA spots revealed an inhomogeneous signal distribution within the spot (Fig. 4). Especially for GOPS surfaces, we found an inhomogeneous distribution of the fluorescence signal; the most intensive signal on the droplet edges (Fig. 4A). But also the spot center showed inhomogeneous signal intensities.

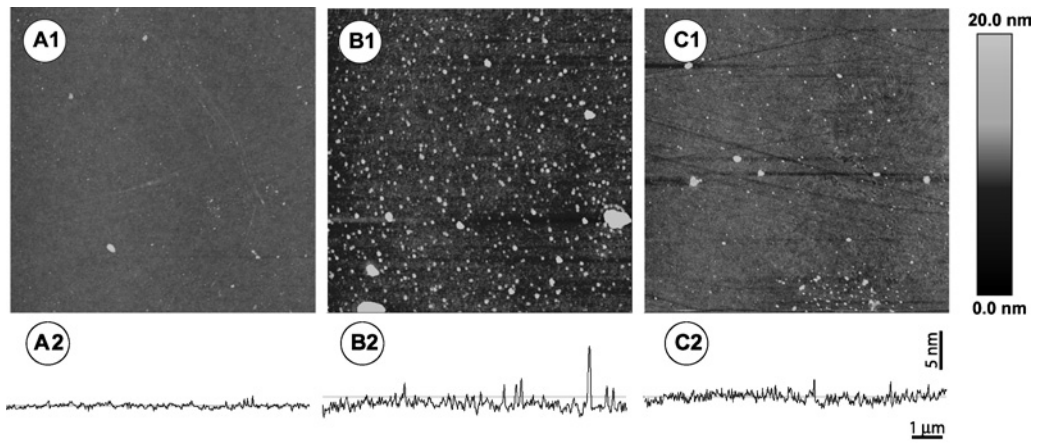

Fig. 2. Characterization of cleaning and silanization methods by AFM. (A1–C1) The AFM pictures (5×5 μm: A; 10×10 μm: B, C) visualize the surface roughness of glass substrates: (A1) precleaned, and after the silanization (B1) 6 hr in 10 mM GOPS/toluene and (C1) 1 hr in 1% APTES/95% acetone/dH_2O. (A2–C2) The section analyses reveal a surface roughness below 1 nm, about 4 nm, and about 2 nm, respectively.

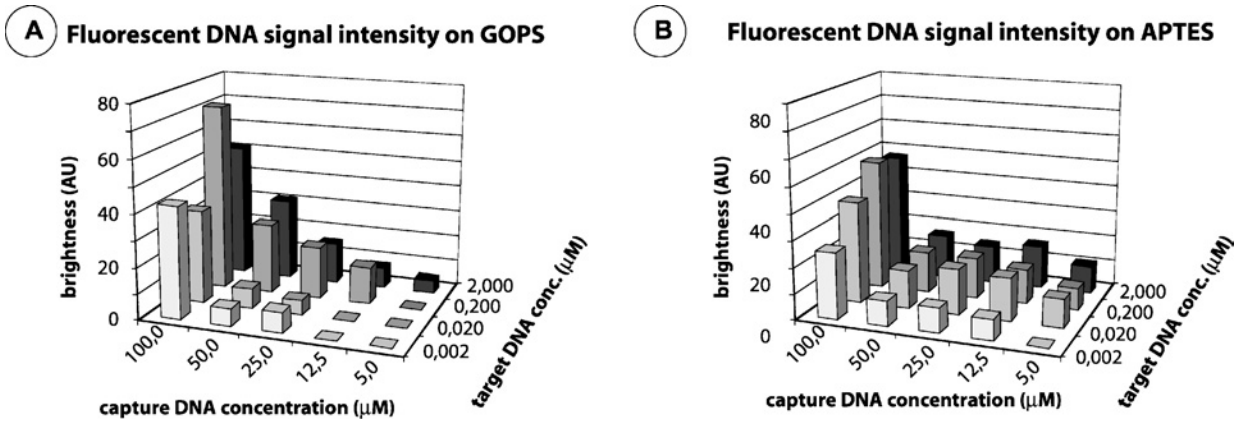

Fig. 3. Comparison of DNA spot intensities for different DNA attachment chemistry. Glass slides (26 × 76 mm) were silanized with (A) GOPS and (B) APTES/PDC, spotted with 0.5–100 μM capture oligonucleotides, hybridized with 0.002–2 μM FITC-labeled complementary oligonucleotides and detected by a microarray reader. The signal intensities were plotted as relative brightness values. Although GOPS exhibited higher maximum fluorescence signals, on APTES also lower capture oligonucleotide concentrations could be detected.

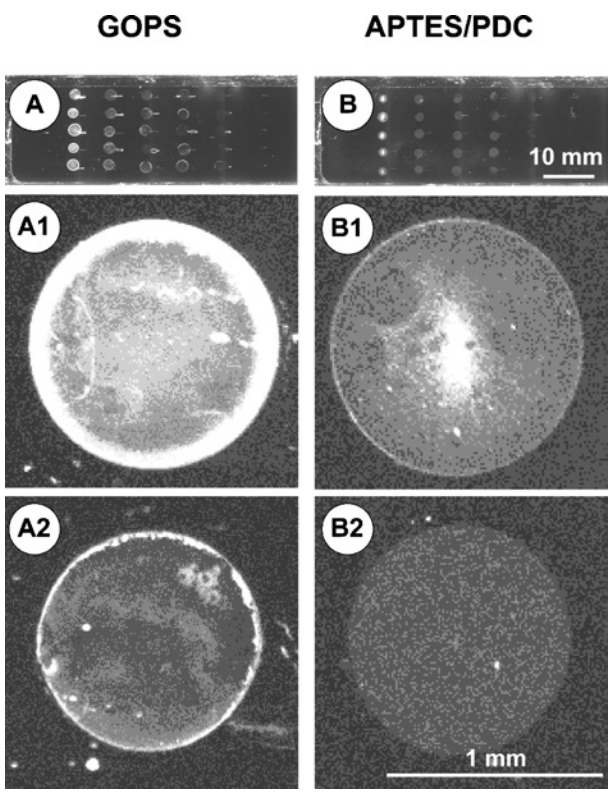

Fig. 4. Characterization of DNA binding homogeneity for different DNA attachment chemistry. (A, B) Overview of GOPS- and APTES/PDC-silanized glass slides, hybridized with 0.2-μM FITC-labeled oligonucleotides. Below, individual spots of 100 μM (A1, B1) and 50 μM (A2, B2) capture oligonucleotides are enlarged. On GOPS, the most intensive signal was on the droplet edges. On APTES/PDC, the signal appeared rather homogeneous, except for high capture DNA concentrations (100 μM).

This phenomenon was observed for both high and low signals. On APTES surfaces, only for high capture DNA concentrations (100 μM) was the distribution of inhomogeneous signal visualized (Fig. 4B). Here, the most intense signals were found in the center of the droplets. For lower concentrations the spots were uniform, pointing to a homogeneous distribution of DNA binding groups on APTES/PDC. The same phenomena were observed with direct fluorescence-labeled capture oligonucleotides excluding the influence of the hybridization process (data not shown).

Blocking of Nonspecific DNA and Gold Binding

Because of this disadvantageous signal behavior (cf. Fig. 4A) and the required elaborate preparation of dry toluene for GOPS silanization, we took the surface modification with APTES/PDC into account for further experiments. Glass stripes (4 × 26 mm) were first silanized in APTES and treated with PDC. For an investigation of the specificity of the DNA detection method, we spotted complementary, partially complementary, and noncomplementary DNA sequences (Fig. 5A). Then the glass stripes were hybridized with gold nanoparticle-tagged DNA solution. For faster and easier analysis, the bound gold particles were enhanced with silver acetate and imaged by a flatbed scanner.

To elucidate the type of chemical bonds between the amino-modified capture DNA and the silane layer, we tried to block the thiocyanate groups of the APTES/PDC surface with ethanolamine before spotting the capture

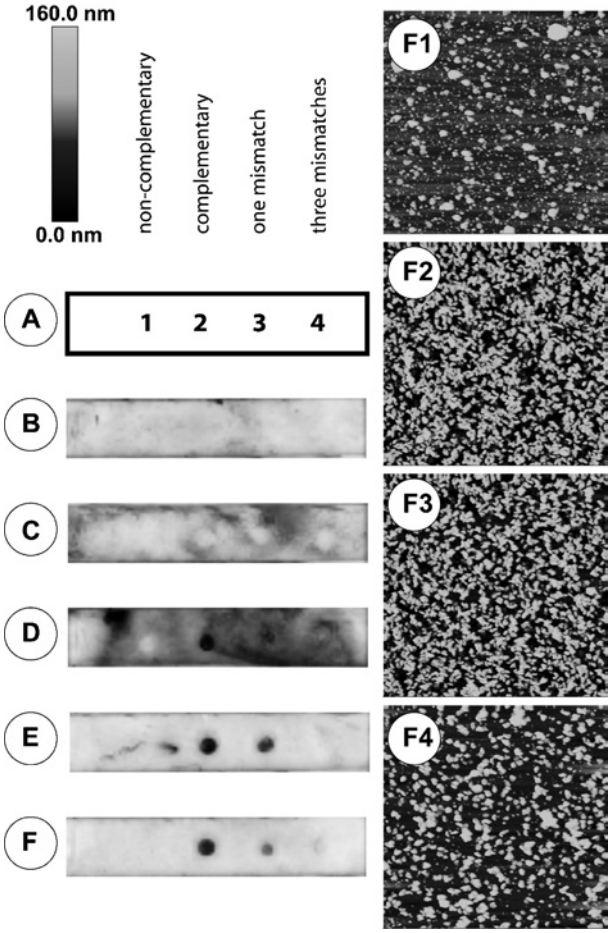

Fig. 5. Binding of gold nanoparticle-labeled target DNA after silver enhancement, and the effect of different blocking strategies against nonspecific DNA and gold binding. (A) Scheme of the microarray substrates (4 × 26 mm glass stripes) with different capture DNA (20 μM) sequences (1–4). (B) Blocking the APTES/PDC-silanized surfaces with ethanolamine anticipates DNA immobilization and nonspecific gold binding. (C) The lack of PDC inhibits the covalent binding of capture DNA on APTES. (D) Usually, treatment with gold nanoparticle-solution leads to nonspecific gold binding on the APTES/PDC-silanized surface. (E, F) The treatment of the silanized, capture DNA-immobilized substrates with BSA/PBS pH 7.4 (1 and 3%, respectively) prevents nonspecific gold binding, without affecting DNA hybridization. (F1–F4) The AFM pictures (10 × 10 μm) visualize the particle densities depending on the different capture DNA sequences.

DNA and hybridization with gold-labeled target DNA. Ethanolamine seemed to hamper DNA immobilization and nonspecific gold binding (Fig. 5B). The amino-groups of the blocking reagent were considered to block the sulfur atoms of the thiocyanate from reacting to the amino-modified DNA. A further indication for specific attachment via the amine modification was addressed by omission of the PDC as a bifunctional crosslinker (Fig. 5C). The lack of PDC inhibited the covalent binding of amino-modified capture DNA on APTES because the two amino-groups should not be able to couple to each other.

Subsequent experiments showed strong nonspecific binding of the gold nanoparticles to the APTES/PDC-silanized surface (Fig. 5D); probably on the one hand by the well-known binding of gold to thiols, and on the other hand by electrostatic interactions between still available positive aminosilane-groups and negatively charged gold particles and/or DNA. Therefore, we tried to passivate the silanized surface against nonspecific gold attachment.

Neither the blocking of free SCN groups with 1% ammonium hydroxide, as described for fluorescence on APTES/PDC surfaces [15], nor the blocking strategies for GOPS (50 mM ethanolamine) did have any effect. In addition, the treating of the surfaces with 6-mercapto-1-hexanol [16], thought to block the left available amino-groups from APTES, was not able to reduce nonspecific gold binding. So we tried BSA as a well-known blocking reagent. After DNA immobilization and washing, the glass stripes were treated with BSA up to 3% in PBS pH 7.4 for 2 hr prior to hybridization [16]. The silver enhancement revealed blocking effects for 1 and 3% BSA/PBS (Fig. 5E,F). The optimum BSA concentration was found to be 3% BSA in PBS pH 7.4 resulting in dark spots on a bright background. The different DNA capture sequences with its different grey values were still distinguishable. For more detailed information about the specifically hybridized nanoparticles, we imaged the different DNA spots by AFM (Fig. 5F1–F4). The highest particle density was found for the complementary sequence (Fig. 5F2). Introduction of one mismatch hardly led to reduced gold-labeled DNA binding whereas three mismatches considerably decreased the number of bound particles (Fig. 5F3,4). A similar low extent of gold binding was observed for noncomplementary capture DNA (Fig. 5F1) pointing to nonspecific gold and/or target DNA binding.

DISCUSSION

Microarray-based DNA detection methods are promising tools for fast and highly parallel DNA detection. They are based on different capture probes, immobilized on the substrate surface. Thus DNA capture probes, so immobilized, are then hybridized with labeled target molecules prior to detection. For a quantification of the signal, the detected signal should reflect the target concentration in solution in a reproducible manner.

Homogeneous functionalized surfaces are a prerequisite for reproducible attachment chemistry allowing sufficient available binding groups and therefore equal binding rates. For this reason we characterized two different surface modification methods regarding their DNA binding and hybridization properties.

Glass is a well-established and suitable substrate for microarrays. The smooth surface and the accessible silanol groups provide ideal conditions for DNA coupling chemistry. For enabling unhindered reaction, the surface has to be cleaned from all organic and inorganic contaminations. The different cleaning strategies, containing organic solvents and strong acids, led to hydrophilic surfaces illustrated as contact angles at 20° and below. The corresponding AFM pictures revealed very smooth surfaces without any contaminations. The substrate modification with organosilanes should lead to a considerably more hydrophobic surface with increased contact angles. The detected surface roughness of 2–4 nm pointed to only a few and homogeneous silane layers, whereas the water sensitive GOPS silanization probably led to some hydroxylated silanes, observed as higher surface roughness.

But both analyses measuring the wettability and AFM roughness could only be regarded as an indication of successful silanization procedure. For the characterization of the hybridization efficiency we hybridized increasing concentrations of capture probes with different concentrations of fluorescent target oligonucleotides. The obtained higher maximum fluorescence signals for GOPS than for APTES/PDC surfaces could be due to a higher density of binding groups on the epoxy surface, supported by the slightly increased surface roughness on GOPS, leading possibly to higher binding capacity. Interestingly, the highest concentration of the fluorescence-labeled probe (2 μM) did not yield the highest signal intensity. We assume that the second highest target probe concentration (0.2 μM) was sufficient to saturate all the available capture probes on the surface. A higher amount of fluorescence-labeled molecules did not increase the number of hybridized target probes (APTES) or seemed to even affect the hybridization (GOPS), potentially by steric hindrance.

A closer look at the DNA spots revealed an inhomogeneous signal distribution within the spot. On GOPS surfaces the most intensive signal was found on the droplet edges. This effect could be found for all the capture DNA concentration whereas on APTES/PDC the signal appeared homogeneous, except for high capture DNA concentrations (100 μM). The same phenomena were observed with direct fluorescence-labeled capture oligonucleotides excluding the influence of the hybridization process. The so-called "doughnut effects" on GOPS is well known and reflects a variation of the DNA concentration in the spot [19]. Because of the increased evaporation on the droplet edges, there is a molecule diffusion/circulation to the droplet border where the molecules then could bind in a higher amount. On APTES/PDC, the immobilization protocol avoided droplet drying. This could be a reason for the lack of this phenomenon. Additionally, the homogenous signal distribution could be facilitated by the smoother surface of the APTES/PDC layer. The opposite effect (for higher concentrations) is unclear. One possible explanation is a faster binding reaction which prevented the molecule diffusion to the edges. Other sources of inhomogeneous signal distributions include the printing process or the postprocessing of the substrates after printing (e.g. insufficient rehydration time) [20]. To overcome these disadvantages, image analysis methods were developed [20,21]. Additionally, Fare *et al*. described deteriorating effects of atmospheric ozone on microarray data quality [22]. The homogeneous signal distribution on APTES/PDC could also be a reason for the higher sensitivity for lower capture DNA concentrations.

While schemes such as Fig. 1 are often presented, there still has to be evidence to confirm them. Contact angles measure mainly hydrophobicity and detect only changes in surface modification. In the case of APTES/PDC, we therefore tried to confirm the covalent coupling strategy via silane groups to glass by chemical controls. APTES silanization provides amino-groups on the surface which have to be crosslinked to attach amino-modified DNA. This crosslinking of the two amino-groups could be performed by PDC as a homobifunctional crosslinker with thiocyanate-groups on both sides. By leaving out this linker the two amino-groups should not be able to couple to each other. We were able to demonstrate this in experiments shown in Fig. 5C where no characteristic DNA spots were visible. Another strategy was blocking the APTES/PDC surface with ethanolamine before addition of oligonucleotides. The amino-groups of the blocking reagent were considered to block the sulfur atoms of the thiocyanate from reacting to the amino-modified DNA. Confirming this suggestion, in Fig. 5B no DNA spots could be seen.

Beside this indication for specific DNA attachment via the amine modification, we observed extended nonspecific binding of the gold nanoparticle-labeled DNA to the APTES/PDC surface. On the one hand, the amino-groups of the silane layer could be responsible for charge interactions with negatively charged gold particles and/or DNA. It might have been possible that not all of the amino-group from APTES had been saturated by the thiocyanate of the crosslinker PDC. On the other hand, the binding of gold to thiols is well known. In this paper, it was also used for

the covalent modification of gold nanoparticles with thiolated oligonucleotides. Therefore, we examined different blocking strategies after immobilization of the capture DNA. The available amino-groups left from APTES were thought to have been blocked by thiol-groups of mercaptohexanol, but no such effect was to be observed. A second approach was the blocking of the thiocyanate groups of PDC which had not been attached to capture DNA. But neither the treatment with ammonium hydroxide nor ethanolamine inhibited the nonspecific gold binding. The reasons for this could be found in an insufficient reaction of the blocking reagents with the surface groups as well as an introduction of new available functionalities.

However, blocking by BSA as a standard procedure prevented the surface from nonspecific gold binding. It resulted in dark silver enhanced, gold nanoparticle-labeled DNA spots leaving the background bright. We suppose that BSA was able to shield the reactive surface groups or charges against the DNA-gold conjugates. The mechanisms of this blocking effect are unknown and it is still unclear why the various functionalities and charges of the large protein BSA do not attract gold or DNA. Though, for amino-modified slides, it was shown that BSA was able to block unreacted functional groups of printed microarrays with chemistries that have low affinity for DNA [23].

For a closer look at the amount of bound nanoparticles, we visualized the different capture DNA spots by AFM. As expected, the highest particle density was found for the complementary sequence. However, one could not distinguish between the totally complementary and the sequence with one mismatch, although the different particle densities were even visible with the naked eye (Fig. 5F). This result could have been caused by inhomogeneous distribution of the nanoparticles in the micrometer range. Additionally, applying a higher melting temperature and more stringent hybridization conditions could improve the distinction of partially complementary DNA sequences. The three mismatches led to considerably fewer immobilized particles in similar ranges like the noncomplementary sequence. The latter also represented the still detectable nonspecific background signal. The high surface roughness, due to the BSA treatment, hampered the detection of individual particles, but should not be a problem for other read out methods beside AFM.

The application of directly-labeled DNA allows kinetic studies and processes involving elevated temperatures. However, the labeling procedure is elaborated and hardly reproducible. Therefore, other alternatives are interesting. Beside the direct attachment of DNA to gold nanoparticles, the indirect coupling by biotin-streptavidin is possible. Therefore, the capture DNA is hybridized with biotinylated target DNA. On the other hand, biotin molecules can also covalently bind to the plain target DNA after hybridization. In an additional step, biotin is able to bind streptavidin-coupled gold nanoparticles. This method offers the opportunity for labeling many different target sequences with one nanoparticle solution at the same time. Further investigations will be aimed at conferring the silanization and blocking protocol to this biotin-streptavidin system.

ACKNOWLEDGMENTS

We thank E. Birch-Hirschfeld for access to and M. Pfeiffer for assistance with the microarray reader. This work was supported by the DFG (FR 1348/1-4) and the integrated project "Nanocut" in the framework of the "Nanobiotechnology" initiative (BMBF 3006-68301).

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