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Immobilization of Oligonucleotides onto a Glass Support via Disulfide Bonds: A Method for Preparation of DNA Microarrays

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The covalent attachment of disulfide-modified oligonucleotides to a mercaptosilane-modified glass surface is described. This method provides an efficient and specific covalent attachment chemistry for immobilization of DNA probes onto a solid support. Glass slides were derivatized with 3-mercaptopropyl silane for attachment of 5-prime disulfide-modified oligonucleotides via disulfide bonds. An attachment density of approximately 3×10^5 oligonucleotides/μm^2 was observed. Oligonucleotides attached by this method provided a highly efficient substrate for nucleic acid hybridization and primer extension assays. In addition, we have demonstrated patterning of multiple DNA probes on a glass surface utilizing this attachment chemistry, which allows for array densities of at least 20,000 spots/cm^2.

Key Words: **covalent immobilization; oligonucleotide; glass; disulfide bonds; DNA microarray.**

In recent years, high-density miniaturized oligonucleotide arrays have emerged as promising tools for assessing genomic data with a lower cost and higher throughput than the traditional gel-based methods. Such oligonucleotide arrays, or DNA chips, have been applied to genetic mutational scanning (1, 2), molecular bar coding (3), gene expression monitoring (4, 5), and sequencing (6–8). The power of the DNA chips come from the highly parallel, addressable, miniaturized array format that provides significant advantages over traditional gel-based formats in terms of reagent cost, labor, speed, throughput, and operational simplicity. The development of efficient chemistries for the manufacture of spatially resolved, microscale DNA arrays on a solid-support is essential for the realization of the DNA chip technology potential. In most DNA chip applications, the DNA arrays are used to capture or analyze the target sequences and/or detection probes via hybridization reactions alone (1–7) or with subsequent primer extension reactions (8). The reliability and integrity of the hybridization reactions are highly dependent, in addition to the actual base composition of the arrayed oligonucleotides, on the quality and the characteristics of the DNA arrays. In developing a useful and reliable chemistry for producing DNA arrays, the accessibility and functionality of the surface-bound DNA, the density of attachment, the stability of the array, the reproducibility of the attachment chemistry, and the fidelity of the immobilized sequences are all critical.

There have been numerous reports regarding immobilization (9–26) or direct synthesis (27, 28) of oligonucleotides on solid supports, such as glass, silicon, membranes, and polystyrene. Parallel synthesis of oligonucleotides directly onto the solid support by photoactivatable chemistries (27) or standard phosphoramidite chemistries (28) have, thus far, been the most successful approach to manufacturing high-density DNA arrays. Patterning of presynthesized oligonucleotides, however, is preferred for many research applications and low- to moderate-density-array applications required for many diagnostic tests. Methods reported for covalent attachment of oligonucleotides generally involve the use of preactivated solid supports, homo- or heterobifunctional cross-linkers and modified/activated oligonucleotides (9–12, 17, 26). Other methods allow noncovalent immobilization of oligonucleotides via passive adsorption (13) or avidin–

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biotin affinity binding (18). Methods utilizing three-dimensional functionalized polyacrylamide gel pads for end-specific immobilization of oligonucleotides have also been reported (29–32). These gel pad methods offer higher oligonucleotide loading capacities per unit of surface area than do other two-dimensional array methods. When coupled with microdispensing and patterning techniques, such as ink-jet printing (33), robot dispensing, or photolithography/laser patterning methods (34), most of these chemistries can be applied to the preparation of DNA microarrays on a solid support. However, there are major issues affecting the use of presynthesized oligonucleotides for array preparation, including the efficiency and specificity of the chemistry and the complexity of patterning a large number of different oligonucleotide species.

Disulfide coupling chemistry has been used in the ligation of peptide to peptide (37), DNA to DNA (37), peptide to DNA (37), DNA to haptens (36), DNA to paramagnetic beads (15), and DNA to controlled porosity glass (CPG)[2] (35). Here, we report a simple, efficient, and specific attachment chemistry utilizing the disulfide bond for the covalent immobilization of presynthesized DNA probes onto a silane-derivatized glass support. Unlike most of the published methods, which often require preactivation or reduction of the disulfide groups to generate more reactive and unstable sulfhydryl species, this method allows for direct coupling of stable 5′ disulfide-modified oligonucleotides onto mercaptosilane-activated glass surfaces without any pretreatment. In addition, because of the specificity of the thiol/disulfide exchange reaction, side reactions involving other functional groups on the oligonucleotides are minimized. Oligonucleotides attached by this method were demonstrated to provide a highly efficient and specific substrate for nucleic acid hybridization and primer extension assays (8).

In addition, due to the hydrophobicity of the mercaptosilane film, the oligonucleotide is confined within distinct droplets on the modified glass surface after deposition. Therefore, simultaneous patterning of multiple DNA probes can be accomplished by directly dispensing the modified probes onto the mercaptosilane-coated surface with an ink-jet printing device or a syringe-pump robot without the addition of other surface patterning techniques.

MATERIALS AND METHODS

Oligonucleotide Synthesis and Purification

The synthesis of the 5′ disulfide-modified oligonucleotides was performed on an ABI 392 DNA/RNA synthesizer using standard phosphoramidite chemistry. The disulfide linkage was added to the 5′-terminus of the oligonucleotides, using a 5′ thiol modifier, 1-*O*-dimethoxytrityl hexyl disulfide, 1′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)] phosphoramidite (Glen Research, Sterling, VA). After synthesis, the oligonucleotides were purified using either HPLC or oligo purification columns. All of the oligonucleotides were desalted with NAP 5 desalting columns (Pharmacia Biotech, Piscataway, NJ) before use.

Preparation and Silanization of Glass Substrate

Glass slides (Erie Scientific Co., Portsmouth, NH) were immersed in 25% ammonium hydroxide solution (Aldrich, Milwaukee, WI) overnight and then rinsed with running milli-Q water for 10 min and briefly rinsed with anhydrous EtOH (Aldrich). The slides were then immersed in a 1% 3-mercaptopropyl trimethoxysilane (MPTS), 95% EtOH, and 16 mM acetic acid (pH 4.5) mixture for 30 min at room temperature. Following incubation, the slides were rinsed with 95% EtOH/16 mM acetic acid (pH 4.5) once and then cured either under dry nitrogen for overnight at room temperature or in a vacuum oven for 2 h at 150°C.

Immobilization of Disulfide-Modified Oligonucleotides onto Silanized Glass via Disulfide Bonds

The attachment of 5′ disulfide-modified oligonucleotides to glass surfaces was performed via an intermediate mercaptosilane layer using a thiol/disulfide exchange reaction. The 5′-disulfide oligonucleotides were diluted to a concentration of 0.01 to 40 μm in 500 mM $NaHCO_3/NaH_2CO_3$ buffer (pH 9.0) or 0.1 M citric acid buffer (pH 4.5) and arrayed onto the mercaptosilane-coated glass slide surface. Arrayed primers were incubated in a humid chamber for 5 min to overnight, followed by three washes in TNTw (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20).

Radiochemical Determination of Nucleic Acid Attachment and Hybridization Densities

Radioactive labeling and phosphorimaging techniques (38) were used to quantify the oligonucleotide attachment and subsequent hybridization reactions. [α-^{32}P]ddATP (Dupont NEN, Boston, MA) was used for 3′ labeling of the attachment probes and target templates via the terminal transferase reaction, which was carried out according to manufacture's instruction (Life Technologies, Gaithersburg, MD), followed by desalting with NAP 5 columns (Pharmacia Biotech, Piscataway, NJ). Specific activities of the radiolabeled oligos were determined via liquid scintillation counting using an LS 7500 liquid scintillation system (Beckman Instruments, Inc., Columbia, MD). The surface radiochemical density was measured with a Fuji BAS 2000

[2] Abbreviations used: CPG, controlled porosity glass; MPTS, 3-mercaptopropyl trimethoxysilane; DTT, dithiothreitol.

phosphorimager (Fuji Photo Film Co., LTD, Tokyo, Japan). Raw data, photostimulated luminescence intensity/mm^2, were converted to molecules/μm^2 by comparing to standard curves made from a serial dilution of known amounts of the ^{32}P-labeled probes used in the experiments. The data presented here are the averages of, minimally, four replicate points.

For the hybridization experiments, a disulfide-modified and nonradioisotope-labeled probe was immobilized on glass, as described above. A 1.0 μM solution of radiolabeled probe was used to hybridize with the surface-bound probes for 30 min at room temperature in 1.5 M NaCl, 10 mM EDTA, and 1 mM cetyltrimethylammonium bromide. The level of DNA hybridization was quantified as described above.

Patterning of Oligonucleotides

Hamilton robot patterning. Arrays, consisting of three different 3′ dye-labeled, 5′ disulfide oligonucleotide species were patterned using a Microlab 2200 robot (Hamilton, Inc., Reno, Nevada). Oligonucleotides were aspirated from 96-well source plates and deposited onto mercaptosilane-coated slides with droplet sizes of 50 nl. In each printed array, the diameter of a 50-nl spot was approximately 1 mm and the spot center-to-center spacing 1.5 mm.

Ink-jet printing. A 5′ disulfide/3′ fluorescein-modified DNA probe was arrayed using an ink-jet printing device (MicroFab Technologies, Inc., Plano, TX) based on the chemistry described above. A single-probe piezoelectric head, with a frequency of 200 MHz, was used to create minimal droplet sizes of approximately 36 pl.

Direct Fluorescent Detection of Signals

For direct fluorescent measurement of oligonucleotide attachment reactions, the slides were scanned using either a fluorescent imager, FMBioII (Hitachi, San Bruno, CA) or a confocal fluorescent scanner (General Scanning, Inc., Watertown, MA). The excitation and detection wavelengths selected were based on the dye labels and the instrument used. For the FMBioII, an excitation wavelength of 530 nm (YAC laser) was used for detection of Cy3, Cy5, and fluorescein with different emission filters: 585, 650, and 505 nm, respectively. On the confocal scanner, an excitation wavelength of 488 nm (Ar laser) was used with a 530 nm emission filter for fluorescein detection.

RESULTS

The disulfide chemistry for immobilization of oligonucleotides onto a glass support is illustrated in Fig. 1. The characteristics and utilities of this chemistry are demonstrated below.

Thiol Disulfide Exchange Reaction Efficiencies at Different Oligonucleotide Concentrations and pH Conditions

We have tested the immobilization of oligonucleotides through thiol disulfide exchange reactions (Fig. 1) with combinations of varying oligonucleotide concentrations (ranging from 0.01 to 10 μM) and two pH conditions (pH 4.5 and pH 9.0) to determine the relative attachment efficiency of this method under those conditions. A 5′ disulfide, 3′ Cy3-labeled oligonucleotide 4254.2 (Table 1) was used in this experiment. The results (Fig. 2) show that the attachment efficiencies were higher at pH 9.0 than at pH 4.5.

Additionally, we have tested immobilization of thiol-modified oligonucleotides, under the same conditions, via direct disulfide formation (data not shown). The results show that, at pH 9.0, both disulfide exchange and direct formation methods provided virtually the same degree of attachment efficiency for all oligonucleotide concentrations tested (0.01–10 μM). At pH 4.5, the direct disulfide formation reaction gave slightly higher attachment densities (about 10% higher) when compared to the exchange reaction at pH 9.0. The direct formation reaction, although providing a moderately higher immobilization efficiency than the exchange reaction, requires thiol-modified oligonucleotides that have to be prepared, prior to each use, by reducing the disulfide-modified oligonucleotides with dithiothreitol (DTT) followed by removal of the reducing agent. The disulfide-modified oligonucleotides, on the other hand, are relatively stable and can be stored directly in the pH 9.0 carbonate buffer for at least 3 months (data not shown) without losing reactivity. Based on these results, we have been immobilizing oligonucleotides using the thiol/disulfide exchange reaction at pH 9.0 and all subsequent tests reported below use this chemistry.

Specificity and Density of Oligonucleotide Attachment

To determine the density and specificity of covalent oligonucleotide coupling via the disulfide exchange chemistry, ^{32}P-labeled oligonucleotides, 4256 and 2982 (Table 1), were attached to a sulfhydryl-modified glass surface as described under Materials and Methods. Reactions were performed with oligonucleotide concentrations ranging from 1–40 μM. Quantitative measurements of the attachment density were obtained using phosphoroimaging as described under Materials and Methods. The results (Fig. 3A) show that, at lower oligonucleotide concentrations, the attachment efficiency is directly proportional to the concentration of the disulfide oligo. The attachment density reaches a plateau with an oligonucleotide concentration of approximately 20 μM, reaching a maximal attachment density of approximately 3.0×10^5 molecules/μm^2. No

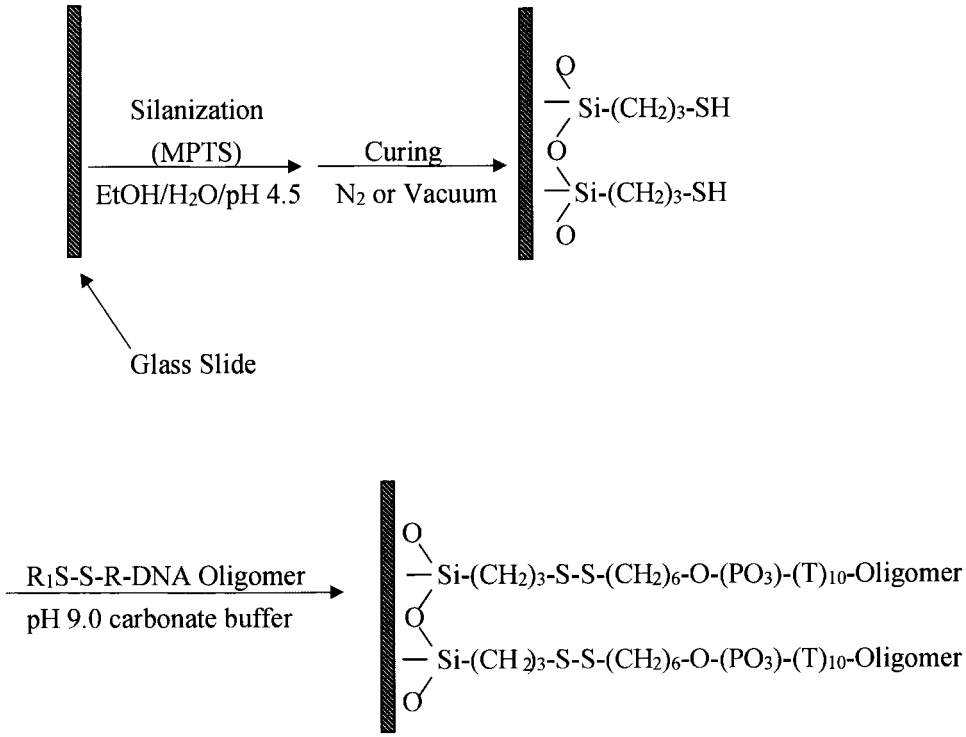


FIG. 1. Attachment of oligonucleotides onto a glass slide via disulfide bonds. The glass slide was first silanized with 3-mercaptopropyl trimethoxysilane (MPTS) and cured before attachment as described under Materials and Methods. The 5′ end disulfide-modified oligonucleotide probes were then immobilized onto the glass slides through a thiol disulfide exchange reaction between the disulfide oligonucleotide and the sulfhydryl group of the mercaptosilane film on the glass slides. R, $-(CH_2)_6$-O-(PO_3)-$(T)_{10}$; R_1, $-(CH_2)_6OH$.

measurable attachment signal was detected from the non-disulfide-modified control oligonucleotide for all concentrations tested.

Disulfide Immobilization Time Course

In order to evaluate the effect of reaction time on the density of oligonucleotide attachment, a ^{32}P-labeled oligonucleotide 4256 (Table 1) was attached to a sulfhydryl-modified surface as described under Materials and Methods. The reactions were done using a 10 μM oligonucleotide solution over time periods ranging from 5 min to 3 h. Quantitative measurement of the attachment density was done using phosphoroimaging as described under Materials and Methods. The results (Fig. 3B) show that 60% of the maximum observed attachment occurs within 10 min and 80% within 25 min. After 2 h, no significant increase in attachment density was observed.

TABLE 1

Sequences of Oligonucleotide Probes Used in this Study

Oligo name	Sequence 5′ to 3′	5′ Disulfide modification
4254.1	CATTAATGCTATGCAGAAAATCTTAG-Cy5	Yes
4254.2	CATTAATGCTATGCAGAAAATCTTAG-Cy3	Yes
4254.3	CATTAATGCTATGCAGAAAATCTTAG-FL	Yes
4256	$(T)_{10}$CATTAATGCTATGCAGAAAATCTTAG	Yes
2982	ACACTCTAAGATTTTCTGCATAGCATTAAT	No
4383	GACAGGCAGGGCTGAGGCAGGCTGA	No

Efficiency and Specificity of Hybridization to Disulfide Bound Oligonucleotides

A major concern in oligonucleotide array preparation is the accessibility and specificity of the surface-bound probe for hybridization. Overloading the surface with probes may cause a crowding effect that can lower the accessibility of the surface-bound probes for hybridization with the targets. In order to evaluate the accessibility and specificity of the surface-bound oligonucleotides for hybridization as a function of surface-bound probe density, ^{32}P-labeled oligonucleotides 2982 and 4383 (Table 1) were hybridized to an array that had been patterned with different densities of oligonucleotide 4256 (Table 1). Oligonucleotide 2982 is a 30-mer synthetic target template that contains a 24-base region, which is fully complementary to 4256. Oligonucleotide 4383 has no complementarity to 4256 and was used as a nonspecific hybridization control. Oligonucleotides 2982 and 4383 were hybridized to the array in a

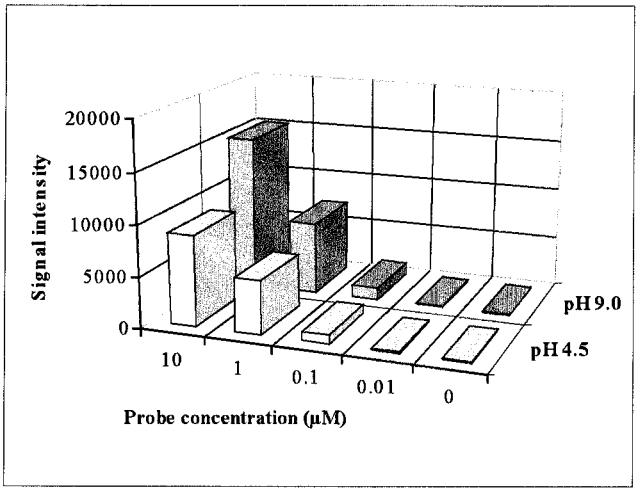


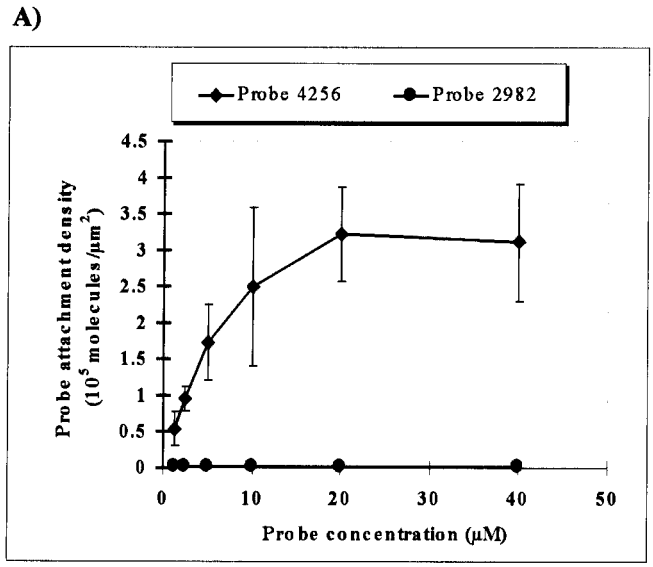
FIG. 2. Attachment of oligonucleotides onto a glass slide through thiol disulfide exchange with different pH levels. The attachment reactions were performed as described in Materials and Methods, except that the reactions were carried out at different pH levels and probe concentrations. Oligonucleotide 4254.2, which was disulfide modified at the 5′ end and labeled with Cy3 at its 3′ end (Table 1), was used in this experiment. The fluorescent signals from the dye label, Cy3, were detected by a FMBioII fluorescent imager with a 530-nm excitation wavelength and a 585-nm emission filter.

total volume of 100 μl at a concentration of 1 μM. The results (Figs. 4A and 4B) show that the hybridization efficiency was directly related to the capture probe attachment density. On average, 16% of the surface-bound probe was hybridized to the target template. No measurable nonspecific hybridization signal from the noncomplementary control template was detected. It was also observed that less than 5% of the disulfide-bound oligonucleotides were detached from the solid support when treated with the hybridization salts used in this experiment for 1 h at room temperature (data not shown).

Patterning of Oligonucleotides

To demonstrate a simple and economical way of producing low- to moderate-density arrays using the disulfide chemistry, a Hamilton Microlab 2200 robot (Hamilton, Inc.) was used to produce an array consisting of three different oligonucleotide species, 4254.1, 4254.2, and 4254.3, each labeled with a different fluorescent dye (see Table 1). Figure 5A shows the patterning results detected by a FMBioII fluorescent imager.

To demonstrate spot size as a function of probe deposition volume and the highest achievable array density using the disulfide chemistry, automated patterning of oligonucleotides by an ink-jet printer was performed by MicroFab Technologies, Inc. Oligonucleotide arrays, with droplet sizes of 125 pl–25 nl and spot diameters of 60 to 360 μm were produced. Figure 5B shows an array printed by an ink-jet printer, using oligonucleotide 4254.3, on a mercaptosilane-coated glass surface where the spot spacing is 250 μm, the droplet size is 2 nl, and the spot size is 140 μm. Figure



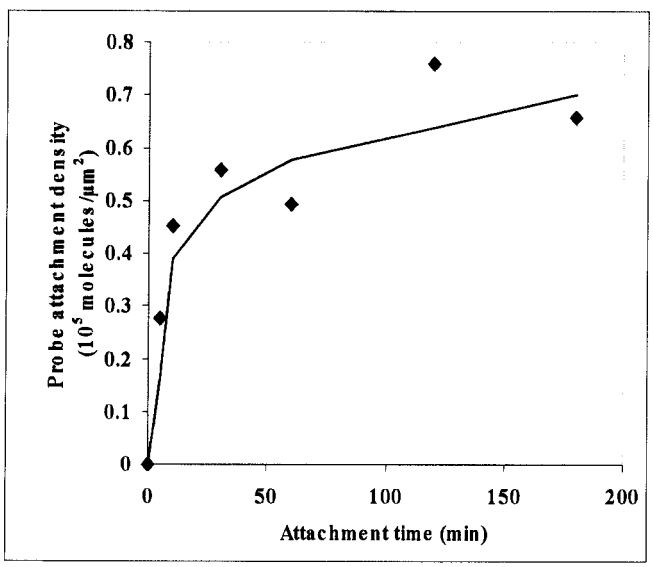


FIG. 3. Attachment density of oligonucleotides onto a glass slide using different probe concentrations and attachment times. Quantitations of the attachment density were presented as the intensity of ^{32}P signals from the probes measured by a phosphorimager as described under Materials and Methods. ^{32}P-labeled 5′ disulfide modified probe 4256 (Table 1) and ^{32}P-labeled non-modified probe 2982 (Table 1) were attached to a sulfhydryl-modified surface via thiol disulfide exchange at different concentrations as described in the Materials and Methods. (A) Attachment densities using different probe concentrations. (B) Attachment densities after different attachment times. Probe 4256, with a concentration of 10 μM, was used for this experiment.

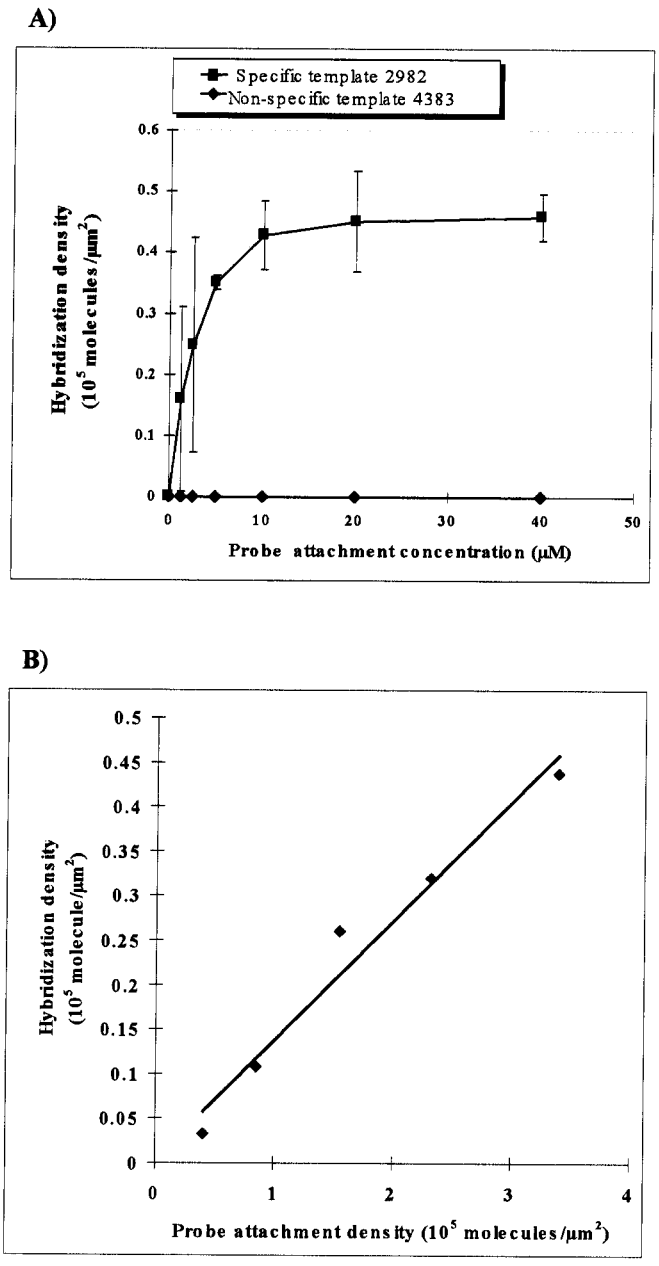


FIG. 4. Efficiency and specificity of the hybridization of a synthetic template to immobilized probes on glass slides. Quantitations of the hybridization density were presented as the intensity of the ^{32}P signal from the labeled templates and measured by a phosphorimager as described under Materials and Methods, except that different concentrations of the 5′ disulfide-modified probe 4256 (Table 1) were attached to a sulfhydryl-modified surface via the thiol disulfide exchange reactions. The attached probe was then hybridized with 100 pmol of ^{32}P-labeled synthetic template 2982 (Table 1) (complementary to probe 4256) and control template 4383 (Table 1) (not complementary to probe 4256). (A) The hybridization of templates to various concentrations of probe oligonucleotide attached onto a glass slide surface. (B) The hybridization densities of template 2982 when hybridized to a probe oligonucleotide (4256) with varying attachment densities on the glass slide surface.

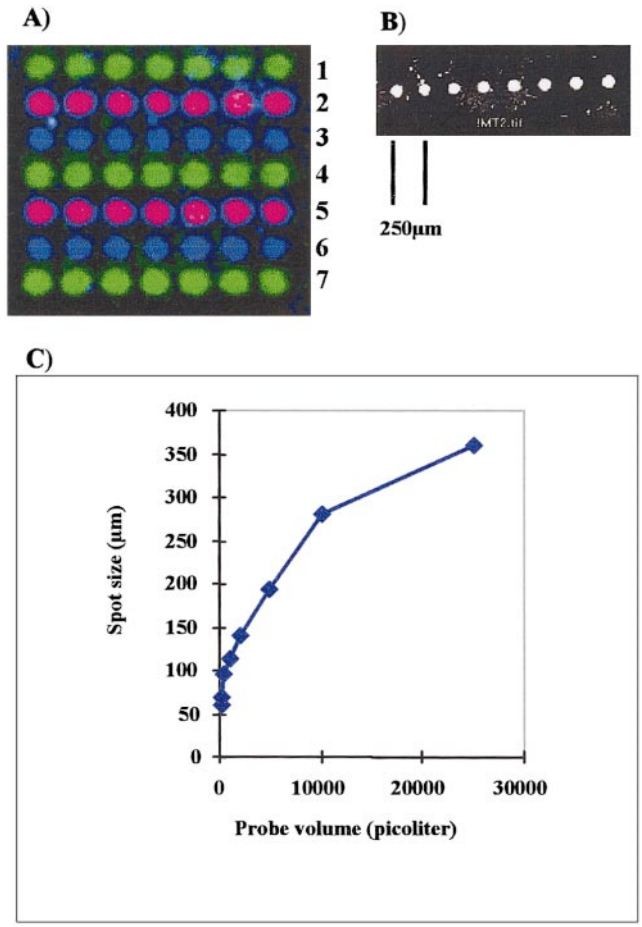


FIG. 5. Patterning of different dye-labeled oligonucleotide probes onto silanized-glass slides using different instruments. (A) Patterning of an array by Hamilton Microlab 2200 robot. Three different oligonucleotides, 4254.1, 4254.2, and 4254.3 (Table 1), each labeled with a different fluorescent dye, were patterned onto silanized-glass slides as a 7 × 7 array. Lines 1, 4, and 7 were fluorescein-labeled 4253.3; lines 2 and 5 were Cy3-labeled 4254.2; and lines 3 and 6 were Cy5-labeled 4254.1. The density of the array is 49 spots/cm^2. The fluorescent signals from the attached oligonucleotide were detected by an FMBioII fluorescent imager at 530-nm excitation wavelength with a 650-nm emission filter for the Cy5 label, a 585-nm emission filter for the Cy3 label and a 505-nm emission filter for the fluorescein label. (B) Automated patterning of an oligonucleotide by an ink-jet printer (MicroFab Technologies, Inc.). The array has spot spacing of 250 μm (center-to-center) and a spot diameter of 140 μm. Probe 4254.3 was used for patterning. (C) Spot size as a function of probe deposition volume by the ink-jet printing method.

5C shows the relationship between the spot size and the probe deposition volume. These results demonstrate that array densities of at least 20,000 spots/cm^2 (assuming 60-μm spot size and 70-μm center-to-center spacing) can be produced by a high-precision and ultra-low-volume microdispenser using this chemistry.

DISCUSSION

Reports have shown that oligonucleotides immobilized through random linkage between the reactive

groups on the nucleic acid backbone or bases (16, 39) and solid supports often result in loss of accessibility for hybridization. On the other hand, stable end-specific covalent linkage-mediated immobilization methods (17, 39–42) have demonstrated increased availability of attached DNA for subsequent hybridization. Therefore, very often functional/active groups are introduced to either the 3′-end or 5′-end of oligonucleotides and solid supports to allow end-specific immobilization. However, these functional groups often cause a high degree of nonspecific binding during attachment (9, 17, 26) and even hybridization (26). For example, it has been reported that nonspecific attachment and nonspecific hybridization of oligonucleotides occurs on both aminosilane-modified (9) and epoxysilane-modified (26) surfaces, which are the two most commonly used silane-modified surfaces for DNA array preparation.

Methods utilizing terminal thiol modifiers for end-specific immobilization of oligonucleotides to fused silicon, silicon, CPG, and paramagnetic beads have been reported previously (9, 15). However, the expected cost and operational complexity of these reported methods is greater than our method, due to either the use of expensive and unstable cross-linking reagents (9) or the addition of reduction/deprotection (15) and activation steps (35). We have demonstrated that the presented disulfide chemistry is very specific, simple, and efficient. It allows for fast, direct coupling of disulfide-modified oligonucleotides, which can be manufactured easily using an automated DNA synthesizer, to mercaptosilane-coated glass slides. Most importantly, no measurable nonspecific attachment was observed, indicating that the fidelity and accessibility of the immobilized oligonucleotides for target template hybridization was preserved without the addition of blocking or stripping steps. In addition, this chemistry gave a relatively uniform oligonucleotide attachment density across each spot (Fig. 5) and did not exhibit the “doughnut” effect (most likely caused by uneven drying of the oligonucleotide droplet) observed for some attachment chemistries (4, 5).

Although the disulfide bond can be cleaved with reducing agents, such as DTT, the attachment is stable between room temperature and 37°C under the hybridization conditions described here. No significant detachment of disulfide-bound oligonucleotides was observed when biological samples and reaction mixes, such as PCR and polymerase extension reaction mixes, were applied (data not shown). Generally, disulfide-linked oligonucleotide-hapten adducts are stable for at least 12 h at 37°C, with the pH between 4.0 to 12.0 (36). As for the stability of printed arrays in storage using this chemistry, it was found that the arrays remained functional for at least 4 weeks if stored under dry and cold (i.e., 4°C) conditions (data not shown). We anticipate that the printed arrays should be stable for a significantly longer period of time, as long as the arrays are not exposed to high humidity, a reducing agent, or extreme high- or low-pH conditions, in which case hydrolysis of the silane layer and the cleavage of the disulfide bonds may occur.

In order for an attachment chemistry to be useful for DNA-array preparation, it has to be compatible with a microdispensing and/or a patterning technique to achieve precise, high-density deposition, localization, and confinement of each oligonucleotide applied. Some chemistries require the use of photolithography techniques to pattern the solid support prior to immobilizing and arraying the oligonucleotides (34). In the cases of the gel-pad methods (29–32), arrays of gel pads must be made before the deposition of oligonucleotides (two runs of patterning and deposition are required) and hydrophobic glass spacers are needed to prevent cross contamination between pads. The disulfide chemistry reported here utilizes a highly hydrophobic mercaptosilane film for immobilization. The hydrophobicity of the silane film confines the deposited aqueous oligonucleotide droplets via surface tension effects and prevents mixing of the individual spots, thus obviating the necessity for additional surface patterning techniques. Therefore, multiple probes can be patterned simultaneously on the surface using a simple robotic liquid-delivering system or ink-jet printing device, with no cross contamination problems between different probes, even in a high-density (20,000 spots/cm^2) array format. An array-manufacturing process employing this chemistry can easily be automated and scaled up using an off-the-shelf syringe-pump robot, micro-spotting device, or ink-jet printing instrument, as well as scaled down, using a hand-held pipette.

Currently, we are investigating issues concerning the efficiency, variability, and stability of this chemistry for large-scale array production. Preliminary results show that the substrate (glass) cleaning methods, the silane deposition mechanism, and the curing procedure all have effects on the efficiency and reproducibility of the attachment chemistry.

CONCLUSION

In this paper, we have presented a simple and effective end-specific chemistry that allows covalent attachment and patterning of oligonucleotide arrays on standard microscope slides via disulfide bounds. We think that the proposed chemistry provides an efficient and inexpensive method for both research and large-scale DNA-array preparation.

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