Characterization of an inexpensive, nontoxic, and highly sensitive microarray substrate

Martin Dufva, Sarunas Petronis, Louise Bjerremann Jensen, Claudia Krag, and Claus B.V. Christensen


An agarose film has been proposed as an efficient substrate for producing microarrays. The original film preparation procedure was simplified significantly by grafting the agarose layer directly onto unmodified microscope glass slides instead of aminated glass slides, and the blocking procedure was replaced with a wash in 0.1× standard saline citrate (SSC) and 0.5% sodium dodecyl sulfate (SDS) without decreasing the performance of the produced microarrays. Characterization of the grafted agarose film using atomic force microscopy (AFM) and scanning electron microscopy (SEM) showed that the agarose film had a 10-fold increase in surface roughness compared to glass and that the interior of the agarose film was porous, with pore sizes between 100–500 nm. A comparison of hybridization on aldehyde-activated agarose-coated microarray slides and commercial amino-reactive microarray slides showed that aldehyde-activated agarose-coated slides had the highest signal-to-noise ratio of 850, suggesting that the aldehyde-activated agarose microarray slides are suitable in applications where analytes have a wide concentration range. By immobilizing the DNA probes using ultraviolet (UV) light, the signal-to-noise ratio was further increased to 3000 on the agarose microarray slides. The specificity of the UV cross-linked DNA probes was demonstrated using 21 and 25 bp long capture probes, enabling discrimination of target molecules differing in only one base.

INTRODUCTION

Glass is the most widely used substrate for the fabrication of DNA microarrays because it is inexpensive, chemically inert, and can be efficiently modified by silanes. Depending on the silane functionality, the resulting surface can have a protruding amine, aldehyde, epoxy, or thiol group, onto which modified or unmodified DNA can react (1–5). The drawback of glass is that the surface is flat, which results in sterical limitations of hybridization reactions (6–8). Dendrimeric linkers have been utilized to increase the binding capacity of glass (2,9,10) and as spacers to elevate the DNA probes from the surface, which increases the hybridization efficiency (7). Polyacrylamide or agarose polymers have been used as alternatives to dendrimeric linkers, showing similar high probe binding capacity and high hybridization efficiency (11–13). Agarose film-coated slides are simple to make and provide reactive aldehyde groups to which amino-modified DNA can bind. Despite limited knowledge about the structure and surface properties of the agarose, it appears that it can be a very efficient hybridization surface compared to aldehyde-modified glass (11). Furthermore, the agarose film was shown to be a highly efficient substrate for single nucleotide polymorphism (SNP) detection using molecular beacon probes (14). Besides enabling rapid and specific hybridization, the agarose film quenches the unhybridized molecular beacon probes better than the corresponding aldehyde-modified glass surface (14). In addition to DNA analysis, the agarose film substrate performed favorably to commercially available substrates in an analytical microarray-based competitive immunoassay for quantifying pesticides in water (15).

Here we describe an inexpensive procedure to fabricate agarose film-coated microarray slides using standard glass microscope slides and an efficient protocol to attach DNA to the agarose film using ultraviolet (UV) light.

MATERIALS AND METHODS

Preparation of Agarose Film-Coated Slides

Agarose film was grafted onto unmodified glass microscope slides and aminated glass microscope slides. Microscope slides coated with activated agarose film containing aldehyde groups were fabricated essentially as previously described (11), with the following modifications. UltraPure™ Electrophoresis-Grade Agarose (Invitrogen, Paislay, UK) was mixed with Milli-Q® water (Millipore, Bedford, MA, USA) to a final concentration of 1% (w/v) and heated in a microwave oven until the agarose was dissolved completely. NaOH (Fluka, Buchs, Germany) was added to a final concentration of 1% (w/v) and heated in a microwave oven until the agarose was dissolved completely. NaN₃ (Fluka, Buchs, Germany) was added to a final concentration of 10 mM, and the mixture was boiled again. One milliliter aliquots of the hot (>65°C) agarose solution were spread onto unmodified Superfrost™ microscope slides (Menzel Gläser, Braunschweig, Germany). After solidi-
fying, the agarose-coated microscope slides were submerged in Milli-Q water for 3 h. The slides were subsequently dried overnight at room temperature, 37°C, or at 50°C until they were completely dry. Slides containing unactivated agarose films were fabricated as described above, with the omission of INaO₄ in the gel solution.

**Preparation of Microarrays**

DNA (Table 1) was spotted using a split-pin (SMB3; TeleChem, Sunnyvale, CA, USA) controlled by a QArray™ robot (Genetix, New Milton, Hampshire, UK) onto six types of microarray substrates: (i) agarose film-coated glass slides prepared as described above; (ii) 3D-Link™ (SurModics, Eden Prairie, MN, USA); (iii) CreativeChip™ Oligo slides (Eppendorf, Hamburg, Germany); (iv) Euray™ Immobilizer Microarray slides (Exiqon, Vedbæk, Denmark); (v) SpotOn™ slides (SMB, Farum, Denmark); and (vi) aldehyde glass microarray slides (CEL Associates, Pearland, TX, USA). The DNA was diluted in 150 mM sodium-phosphate buffer, pH 8.3, to a final concentration of 20 μM if not mentioned otherwise. The DNA was allowed to immobilize overnight in a salt chamber (agarose, 3D-Link, Euray, SpotOn slides) or in a dark and dry chamber (aldehyde and CreativeChip slides). Alternatively, the DNA was immobilized by irradiation at 254 nm UV light for 3 min using a Stratalinker® 2400 (Stratagene, La Jolla, CA, USA) if not mentioned otherwise. The glass aldehyde microarray slides were blocked using NaBH₄ as previously described (16). The 3D-Link, SpotOn, Euray, and CreativeChip microarray slides were blocked according to the respective manufacturers’ instructions. The agarose-coated microarray slides were blocked if not noted otherwise by washing the slides in 0.1× standard saline citrate (SSC; Promega, Madison, WI, USA) supplemented with 0.5% (w/v) sodium dodecyl sulfate (SDS; Sigma-Aldrich, Steinheim, Germany) for 10 min, followed by a 5-min wash in 0.1× SSC. Finally, the slides were rinsed with distilled water and centrifuged or dried using a gentle flow of N₂ gas.

**Hybridization and Quantification of Spots**

DNA target was diluted in hybridization solution (5× SSC, 0.5% SDS, if not mentioned otherwise) to a 10-nM final concentration. The microarrays were hybridized with the target under a coverslip for 1 h at 37°C, which saturates the spots. The coverslips were removed, and the microarrays were washed at room temperature for 10 min in 0.1× SSC, 0.5% SDS, then for 5 min with 0.1× SSC, and finally rinsed in distilled water and dried as described above. The microarrays were scanned in a ScanArray™ Lite (Packard Biosciences, Billerica, MA, USA), with appropriate settings to avoid saturation of the signals. The spots were quantified in ScanAlyze version 2.5 (17) from the 16-bit grayscale images generated by the ScanArray software. In some cases, the signals were normalized to adjust for scanning with different sensitivity settings. The normalization was possible because a simultaneous change in sensitivity settings of the scanner followed the empirically found relationship I = 5e⁻²¹S¹.₄₄₁ (r = 0.9999), where I is the scaling factor and S is the scanner settings in percent.

Target-Probe hybrid melting curves were obtained by washing hybridized microarrays in 0.1× SSC, 0.5% SDS for 10 min at different temperatures ranging 25°C–55°C, in steps of five degrees before scanning the microarrays. The melting temperature (Tₘ) was calculated using the formula: Tₘ = 81.5 + 16.6(Log₁₀[Na⁺]) + 0.41(fraction G + C) - 600/N where N is the chain length (18).

**Morphological Characterization of Agarose Slides**

The micro-architecture of the agarose films was investigated using a LEO 1550 field emission scanning electron microscope (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany). Low acceleration voltage (2–5 keV) and glancing incident angles (20°–40°) of the probing electron beam helped to prevent image distortion due to charging of the nonconductive agarose films. A Dimension 3100 Scanning Probe Microscope equipped with a metrology atomic
force microscopy (AFM) head (Digital Instruments, Santa Barbara, CA, USA) was used to quantify the roughness of the agarose film and unmodified glass slides. The surfaces were imaged in tapping mode with a relatively high amplitude (to avoid sticking) using NSC12 silicon probes (MikroMasch, Portland, OR, USA) at 286.845 kHz tapping frequency. The AFM images were analyzed using NanoScope® software version 5.12r3. Several surface roughness parameters, such as mean roughness ($R_a$), root-mean-square roughness ($R_{rms}$), maximum height ($R_{max}$), and three-dimensional (3-D) surface area ($R_{area}$), as described by Stout et al. (19), were calculated and evaluated for quantitative topographic characterization of the bare glass surface and the agarose coating.

A stylus profilometer (Tencor Profiler; KLA-Tencor, San Jose, CA, USA) was used to assess the thickness of the agarose films. Nine measurements were performed in the peripheral and central parts of the agarose film-coated glass microscope slides, testing three microscope slides in total.

RESULTS

Surface Analysis of Activated Agarose Film-Coated Slides

Microscope slides coated with agarose film were subjected to SEM and AFM analysis. The surface of the agarose film appeared to be almost 10-fold rougher than the glass substrate (when comparing $R_a$, $R_{rms}$, and $R_{max}$ parameters), exposing an apparent hill-like structure. Although the surface appeared rough, the 3-D surface area of the agarose film was calculated to be only 5% larger than on the equivalent glass surface area. SEM analysis of a cross-section of the film showed a porous, sheet-like structure (Figure 1). The size of the pores was estimated to be 100–500 nm, with orientation predominantly parallel to the surface.

Although the agarose films were prepared by manually applying the agarose solution to the microscope slides, the thickness of the dried agarose films was relatively uniform, with an average film thickness of 6.9, a 0.9 standard deviation between the slides, and a 0.6-μm deviation within a single slide.

Stability of the Agarose Film During Hybridization

Originally, the activated agarose film was grafted onto amino-modified glass (11). The amines on the glass surface presumably formed bonds to aldehyde groups in the agarose, which may

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**Table 1. DNA Oligonucleotides**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>LucT</td>
<td>5'-GCCGCCGAGGATTGTTTGTGGACGAAGTACCAGAAAGGCTTTACCAGGAAACTCGACGC-3'</td>
<td>5' Cy™3</td>
</tr>
<tr>
<td>LucPAm</td>
<td>5'-GCCGTCAGCTTTCCGTAAGCATTCCTTGGACTCTTCACCACAAACACACACTCTCCGCGG-3'</td>
<td>3' amino</td>
</tr>
<tr>
<td>LucP</td>
<td>5'-GCCGTCAGCTTTCCGTAAGCATTCCTTGGACTCTTCACCACAAACACACACTCTCCGCGG-3'</td>
<td>None</td>
</tr>
<tr>
<td>W54P</td>
<td>5'-ATTGCGACGTTGGAAGATGCTGCCACGTCATCGACAAAGCTTGTCTCAGGAACTCATC-3'</td>
<td>N.A.</td>
</tr>
<tr>
<td>W54PAm</td>
<td>5'-ATTGCGACGTTGGAAGATGCTGCCACGTCATCGACAAAGCTTGTCTCAGGAACTCATC-3'</td>
<td>5' amino</td>
</tr>
<tr>
<td>W54(wt)T</td>
<td>5'-GATGACTTCGCAGAGCTTTGCTGATGAAGCTGCGACATCTTCAACTTGCGAATT-3'</td>
<td>5' Cy3</td>
</tr>
<tr>
<td>W54(mt)T</td>
<td>5'-GATGACTTGCGAGACAGGCTTGTCAATGAGCGTGAGAGCTCCTCAACTTGCGAATT-3'</td>
<td>5' Cy3</td>
</tr>
<tr>
<td>W54(21wt)T</td>
<td>5'-CAACGCTCATCGACCGTGCAAGCTTC-3'</td>
<td>N.A.</td>
</tr>
<tr>
<td>W54(21nt)T</td>
<td>5'-CCACGCTCATCGACCGTGCAAGCTTC-3'</td>
<td>N.A.</td>
</tr>
<tr>
<td>W54(25wt)P</td>
<td>5'-TGCCACGCTCATCGACCGTGCAAGCTTC-3'</td>
<td>N.A.</td>
</tr>
<tr>
<td>W54(25nt)P</td>
<td>5'-TGCCACGCTCATCGACCGTGCAAGCTTC-3'</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

LucT, LucPAm, LucP, W54P, W54PAm, W54(wild-type, wt)T, and W54(mutant type, mt)T were all high-performance liquid chromatography (HPLC)-purified. N.A., not applicable.

The base varied to simulate a single nucleotide polymorphism is denoted in bold.

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![Figure 1. Agarose film on glass surface morphology examined using scanning electron microscopy. Magnification, 16,000x.](image)
explain why these slides could sustain incubation at 55°C for 4 h. Noticing that slab gel agarose attaches strongly to unmodified glass, we examined whether activated agarose film-coated glass slides could withstand hybridization reactions. The results showed that the agarose film grafted to ordinary glass slides could withstand hybridization at 37°C for at least 2 h and 50°C for 1 h without detaching from the glass. These conditions are compatible with hybridization of PCR products for genetic analysis as well as for protein analysis (15). The agarose film appeared as a gel after hybridization at the 50°C temperature, which suggests that the agarose is rehydrated during hybridization. The swelling was not as pronounced at incubations at 37°C or at room temperature (data not shown).

Influence of Different Buffer Composition on Hybridization Signal and Background

A novel blocking procedure was developed because blocking by NaBH₄, as described in the original protocol (11), efficiently detached the activated agarose film from the unmodified glass. To avoid the release of the agarose film, the agarose film-coated slides were preincubated with different blocking solutions with or without salmon sperm DNA and with and without SDS (Figure 2) prior to hybridization. The DNA was diluted in hybridization buffer (with or without SDS) to examine the effects of SDS on the hybridization and background signals. The background signal on agarose film-coated slides after hybridization was slightly higher (approximately 10%) than the corresponding background on unhybridized slides. The addition of SDS to the hybridization or blocking buffer decreased the background level by a factor of two (data not shown). A similar reduction of the background signal on nylon membranes by SDS has been previously observed (20). The background signal after hybridization on agarose film-coated slides was 20% higher than the corresponding background on aldehyde glass slides blocked with NaBH₄. The inclusion of SDS in the hybridization solution increased the hybridization signal more than 20-fold (Figure 2). By contrast, the corresponding increase on the aldehyde glass slides was 4-fold (Figure 2).

The procedure for blocking activated agarose slides was compared with the blocking procedure involving NaBH₄. Agarose film-coated slides (on aminated glass) were blocked with NaBH₄ or a simple wash with 0.1× SSC supplemented with 0.5% SDS. The results showed that there was no significant difference in the signal or the background intensity between the slides blocked with NaBH₄ or 0.1× SSC supplemented with 0.5% SDS (data not shown). Furthermore, there was no difference in the performance between agarose slides fabricated with unmodified glass and aminated glass blocked.
with 0.1× SSC, 0.5% SDS (data not shown), indicating that the agarose layer is solely responsible for the retention of probes that can hybridize to a target.

**Comparison of Hybridization Reactions on Different Amino-Reactive Slides**

Six amino-reactive microarray substrates, including the activated agarose film substrate, were spotted with increasing probe concentrations (between 0.1 and 50 μM) to identify the optimal DNA oligonucleotide spotting concentration. The slides were subsequently hybridized with target DNA (LucT) to evaluate the hybridization performance of the different probe concentrations. The results showed that hybridization signals on all slide types were highest when printing the probes at a concentration of 10 or 20 μM (data not shown), which is similar to results found by others (10).

The hybridization performance of the respective substrates was evaluated by calculating the signal-to-noise ratio after hybridization. The signal-to-noise ratio varied significantly between microarrays, ranking agarose and the CreativeChip slides highest (Figure 3A, signal-to-noise ratio between 600–800). The 3D-Link and SpotOn slides had a 2-fold lower signal-to-noise ratio. However, the performance of the 3D-Link and SpotOn slides was increased to signal-to-noise ratios of 700–800 by spotting the probes diluted in the spotting solution provided with the CreativeChip slides (data not shown). The hybridization conditions, as a function of spotted DNA concentration from three independent hybridizations with each of four replicate spots. The error bars represent the standard error of the mean (SEM). S/N ratio, signal-to-noise ratio; Ald. glass, aldehyde glass slides; Norm. fluorescence, normalized fluorescence.

**Figure 3. Comparison of different microarray substrates in hybridization reactions.** Five commercial microarray substrates and agarose substrates with three different percentages of agarose were compared in terms of signal-to-noise ratio and signal strength after hybridization with a saturating amount of LucT. (A) The graph shows the average signal-to-noise ratio for the respective substrates. The microarrays were scanned at different photomultiplier tube (PMT) and laser power settings, and the signal-to-noise ratio was calculated as the signal divided by two times the standards deviation of the buffer spots. The highest signal-to-noise ratio obtained for each slide is shown in Figure 3A. (B) Signal intensity at maximum signal-to-noise ratio. To compare the different signals obtained at a maximum signal-to-noise ratio, the signals were scaled as described in the text to compensate for different scanner settings. The error bars represent the standard error of the mean (SEM). S/N ratio, signal-to-noise ratio; Ald. glass, aldehyde glass slides; Norm. fluorescence, normalized fluorescence.

**Figure 4. Immobilization of DNA probes to agarose films using ultraviolet (UV) light.** (A) Molecular requirements for linking DNA probes to agarose by UV light. Increasing concentrations of W54PAm, LucPAm, and LucP were spotted onto activated and nonactivated agarose film-coated slides. The slides were either incubated overnight in a humidity chamber (overnight at room temperature) or exposed to UV light for various lengths of time. The graph shows the average relative fluorescence signals from three independent hybridizations with each of four replicate spots. The error bars represent the standard error of the mean (SEM). (B) Average hybridization signals obtained using different immobilization conditions, as a function of spotted DNA concentration from three independent hybridizations with each of four replicate spots. The error bars represent the SEM. Min., minimum; Rel., relative; O/N, overnight; Conc., concentration.
hybridization signals varied between the slides showing the highest hybridization intensities for the 3D-Link and agarose-coated slides (Figure 3B). The discrepancy in the signal-to-noise ratio between agarose film-coated glass slides and 3D-Link slides could be explained by the 2-fold higher signal obtained on agarose film-coated glass slides at these hybridization conditions because the noise level was equal on these two slide types (data not shown). By contrast, the CreativeChip and SpotOn slides had relatively low signals (Figure 3B), but the corresponding noise level was 2- to 3-fold lower than the agarose film-coated glass slides (data not shown), compensating partly for the low signal. There was no large difference in the signal-to-noise ratio obtained from hybridizations to agarose films prepared from 0.25%, 0.5%, and 1% agarose solution, although a slight but significant increase in signal was observed with increased percentage of the gel (Figure 3). This observation and the fact that both 0.25% and 0.5% agarose slides are difficult to fabricate (the 0.25% and 0.5% agarose films are fragile, detach easily from the glass when submerged in water, or crack during drying) led to 1% agarose slides being routinely used.

We compared the probe density and the hybridized density of the microarrays on activated agarose film-coated slides and aldehyde glass slides. The probe density and hybridized density were estimated by comparing the fluorescence signals of the immobilized probes and targets with a corresponding standard curve as previously described (21). Assuming that approximately a 1-nL droplet is deposited in each spot, the probe density was calculated to be 100 fmol/mm² on agarose slides for an aminated 60-bp probe spotted at 20 μM concentration. This result was obtained in three independent experiments utilizing three different standard curves. Correspondingly, the hybridized density on the agarose slides was calculated to be approximately 35 fmol/mm². The probe density values for the aldehyde glass slides were 7 and 5 fmol/mm² hybridized density. This indicates that the higher hybridization density observed on activated agarose film-coated glass slides as compared to the aldehyde-modified glass slides was due to higher immobilization densities of the probes.

**Immobilization of DNA to the Agarose Film by UV Light**

The effect of UV light, aminomodification of the probes, and aldehyde modification of the agarose film on DNA probe immobilization were investigated. Unmodified and aminomodified probe DNA was spotted onto slides coated with activated and unactivated agarose (i.e., agarose films with and without reactive aldehydes). The microarrays were irradiated by UV light to immobilize the probes prior to hybridization. The results showed that the immobilization of probes by overnight incubation was highly dependent on the presence of aldehydes in the agarose film because almost no signal was found on nonactivated agarose film (Figure 4A). The terminal amino group was found to contribute very little to the binding of the 60-bp DNA oligonucleotides to the activated agarose film because no difference was observed in the hybridization signal between the unmodified and the modified oligonucleotide. By contrast, on aldehyde glass slides, the amine group on the LucP probe increased the amount of target captured by a factor of four to five (data not shown). The overnight incubation of the probes on the activated agarose-coated slides resulted in a 3-fold higher signal than slides that were incubated for 10 min after spotting (Figure 4B), indicating that although the spots are dried, there is a time requirement for maximum probe binding. In comparison, the UV light-induced cross-linking resulted in quick and efficient probe immobilization, showing signals that were twice as high as overnight incubation (Figure 4A). The signal-to-noise ratio was calculated to be approximately 1000 for hybridization to probes immobilized overnight by the passive incubation, which is close to the 850 obtained in the previous experiment (see Figure 3A). The corresponding signal-to-noise ratio obtained on activated agarose slides that were UV cross-linked for 3 min was approximately 3000.

A concern when using UV light to cross-link DNA to a solid support is the
identification of conditions at which DNA is efficiently bound while retaining functionality. Three to six minutes (3–6 mJ) of UV light resulted in the maximum hybridization signals, while longer or shorter cross-linking times resulted in less signal (Figure 4A). UV-induced binding of DNA to the agarose film was not solely dependent on the presence of aldehydes in the agarose film because probes could be immobilized on agarose films lacking aldehyde groups, indicating that the DNA, at least to some extent, forms other types of bonds with the agarose film.

The DNA probes were spotted in different concentrations to determine the spotting concentration that resulted in the highest hybridization signals for the different immobilization methods. Maximum hybridization signal for slides that were not UV-irradiated was reached at DNA probe concentrations of 5 μM, whether aminated or not (Figure 4B). By contrast, using UV light, the hybridization signals increased steeply with spotted probe concentration (Figure 4B). Overnight incubation alone also increased hybridization signal with increasing DNA probe concentration, although the increase was less than for the UV-mediated immobilization of DNA (Figure 4B). These results support that UV light exposure leads to higher probe densities than passive incubation.

**Specificity of Unmodified DNA Probes Immobilized with UV Light**

To evaluate whether unmodified probes immobilized to activated agarose film by UV light could be used for SNP detection, a synthetic SNP system was developed in which 21- or 25-bp probes were used to capture W54 targets. Melting points for the hybrids and the ability to discriminate between perfect match and mismatch hybridization were determined for the synthetic SNP system. Figure 5A shows an example of a melting curve for the W54(mutant type, mt)T and the 21- and 25-bp perfect match probe/target hybrids. Figure 5B shows the measured melting points. The results showed that the measured melting temperature was approximately 10°C lower than the calculated value of each probe, indicating that the probe properties might be significantly altered by the immobilization procedure (Figure 5B). The corresponding mismatch hybrid melting temperatures were 4°–5°C lower for the 25-bp probes and 5°–6°C for the 21-bp probes (Figure 5B). In comparison, the wild-type 25-bp probe attached to aldehyde glass via a 5′-terminal amino group had a melting temperature of 40°C for the perfect match and 37°C for the mismatch hybrids. The microarrays were hybridized with W54(wild-type, wt)T (match) DNA, W54(mt)T (mismatch) DNA, or a 1:1 mixture of each probe, simulating hybridization of the target DNA that was homozygote for wild-type, homozygote for mutant, or heterozygote. The results showed that the wild-type to mutant probe signal ratio was close to one at low stringency washing (25°C) irrespective of the target used (Figure 5, C and D). By contrast, washing at higher temperatures resulted in wild-type to mutant probe signal ratios that were increasing for microarrays hybridized with W54(wt)T DNA, while decreasing for microarrays hybridized with W54(mt)T DNA (Figure 5, C and D). A mixture of W54(wt)T and W54(mt)T DNA resulted in a ratio of close to one at all washing temperatures (Figure 5, C and D). The match-mismatch discrimination initiated at 40°C for the 21-bp probe and at 45°C for the 25-bp probe, correlating to the measured melting temperatures. How-
ever, maximum discrimination was obtained at 45°C for the 21-bp probe and at 50°C for the 25-bp probe.

To exclude the possibility that the significant decrease in fluorescent signals by washing the microarrays at 50° and at 55°C was not due to a loss of integrity of the agarose and thus the probes, the fluorescence signal of the W54(wt)/T hybridized to W54P [melting temperature (Tm) = 70°C] was investigated for washing temperatures between 20° and 60°C. The hybridizations showed constant signals up to 55°C, indicating that the decrease in fluorescence signal between the long W54 target DNA and the shorter 25- and 21-bp W54 probes was due to denaturation and not due to a loss of probes on the surface. By contrast, a 4-fold decrease in signal was observed for the 60-bp hybrid by washing the slides at 60°C (data not shown). The uncharacteristically sharp decrease in fluorescence signal and the fact that agarose melts at 60°–65°C suggest that the integrity of the agarose is compromised at 60°C. Taken together, the results demonstrate that unmodified DNA probes immobilized to activated agarose by UV light can discriminate between 1 bp mismatch in a 21- and 25-bp sequence.

DISCUSSION

Grafting agarose film onto ordinary glass microscope slides is a simple, nontoxic, and inexpensive method to produce microarray substrates. The agarose film shows excellent performance in hybridization reactions, yielding high signals combined with a relatively low background signal and low noise levels.

The agarose film may function as a dendrimeric linker that characteristically results in higher hybridization signals compared to planar surfaces (2,9,10). Such notion is supported by the fact that the 3D-Link slides (containing dendrimeric linkers on the surface) and the agarose slides showed the highest signals, while the two-dimensional surfaces, such as the SpotOn, aldehyde glass slides, CreativeChip, and Euray slides, all resulted in lower hybridization signals (Figure 3C). The reason for the high hybridization signals of the agarose film, as compared to the aldehyde glass slides, is that agarose film binds more DNA probes than the aldehyde glass slides. It is currently uncertain where the probes are located on the agarose film. Several observations suggest that the agarose film functions as a nonporous film, at least during the spotting of the probes. One nanoliter droplets of probe solution are printed on the surface and disappear within seconds after the pin has left the slide surface. At least two possibilities exist for the droplet disappearance. The droplet either evaporates or is absorbed by capillary forces into the agarose. The spot geometry suggests that the agarose film is functionally nonporous during spotting. Generally, when spotting with 10–20 μM DNA solutions resulted in round homogenous spots. However, spotting with lower amounts of DNA usually result in typical “coffee ring” spots that can be observed when drying low viscosity liquids on a planar surface (22). Furthermore, compared to the acrylamide gels with co-polymerized DNA probes in the matrix, the agarose films displayed almost 10-fold lower hybridization densities (13). However, the high dependence of SDS on the hybridization signals suggests that hybridization is taking place within the pores because it is likely that SDS would enhance the penetration of water into the pores by reducing the surface tension. Furthermore, a slight but significant reduction of hybridization signal was observed when decreasing the percentage of agarose in the gel prior to film formation (Figure 3), which indicates that there is a dependency of hybridization signals and the amount of agarose on the glass surface. Such an effect is not compatible with the notion that the agarose is a solid surface that would be insensitive to the thickness of the agarose film. Finally, the surface area of the agarose film, as measured by AFM, was only 5% more than on glass slides, indicating that surface area could not explain the 10-fold higher probe and hybridization densities observed on agarose film compared to aldehyde-modified glass.

Unmodified DNA probes could efficiently be immobilized to agarose film either by UV light or by a passive overnight incubation. The nature of DNA to agarose binding is unclear, although it does require aldehyde groups in the agarose film for efficient immobilization (Figure 4), indicating that aldehyde-reactive groups like amines in the DNA are involved in the binding event. We speculate that the DNA is mostly attached to the agarose film through amine groups in the DNA bases because the terminal amino modification did not affect the amount of target captured, at least not in these 60 bp-long probe molecules. Further support to the notion that internal bonds are formed between the agarose film and the DNA probes is the observation that molecular beacon probes could not fully be quenched on agarose or glutaraldehyde glass slides (14). This indicates that a fraction of the spotted probes within a spot (20% in case of agarose film and 60% on glutaraldehyde glass slides) was nonfunctional because it could not form a stem-loop structure that quenches the fluorescent signal upon the addition of MgCl2. Our results suggest that the nonfunctional probes are bound by internal bonds between the DNA and the solid support, which would inhibit the stem-loop formation required for the quenching of the fluorochrome.

The bonds between the DNA and the agarose films induced by UV light most likely change the hybridization properties of the capture probe, as illustrated by the experiments with the 21- and 25-bp capture probes that showed large reductions in melting point compared to the calculated melting point. The almost 10°C decrease in melting temperature corresponds to the average decrease in melting temperature by introducing 2- to 3-bp mismatches. The decrease in melting temperature is most likely due to the destruction of at least one base in the probe and because one or two bases surrounding the base involved in connecting the DNA strand to the agarose film have a decreased affinity for the incoming target due to steric hindrance. Because there is no evidence that certain DNA sequences are involved in the agarose-DNA bond, we currently favor a model that predicts that the bond between the agarose and the DNA can involve any base. Thus, a spot contains a collection of different probes that are more or less able to hybridize. Alternatively, the decrease in the
melting temperatures of the probes is the result of electrical repulsion of the target molecules due to the substrate itself or due to the high concentration of negatively charged DNA in the spot (23,24). These effects may explain the 15°C decrease in melting temperature of the wild-type 25-bp probe connected to an aldehyde-modified glass via a 5’ end terminal bound. Taking into account that the unmodified DNA has to be connected to the agarose film by at least one base, which results in fewer bases of the probes involved in hybridization and thus a decrease in melting temperature, and that the decrease in melting temperature is only 10°C for probes attached to the agarose film by UV light, the electric repulsion effect in the agarose film might be relatively small compared to glass substrates. Despite the changed hybridization properties of the probes, the probes are able to efficiently and reproducibly discriminate between target sequences differing only in 1 bp (Figure 5), indicating that as a collection the attached probes can be used for genotyping. The 21-bp probes had lower melting points than the 25-bp probes (Figure 5B). Furthermore, a 31-bp probe was tested and had melting temperature above 55°C. This indicates that, although the probes melting temperatures are changed by attaching the probes to the agarose film, the changes appear not to be completely random because the relationship between the probe length and the melting temperature appears to be maintained.

In conclusion, the agarose film used as described here is one of the most simple and inexpensive microarray substrates described. Furthermore, short unmodified probes UV cross-linked to the agarose film could discriminate between 1-bp mismatches, indicating that a cost-effective SNP detection system could be built using these methods.

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COMPETING INTERESTS

STATEMENT

The authors declare that they have no competing interests.

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