Synergistic effects of epoxy- and amine-silanes on microarray DNA immobilization and hybridization

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Most microarray slides are manufactured or coated with a layer of poly(L-lysine) or with silanes with different chemical functional groups, for the attachment of nucleic acids on to their surfaces. The efficiency with which nucleic acids bind to these surfaces is not high, because they can be washed away, especially in the case of spotting oligonucleotides. In view of this, we have developed a method to increase the binding capacity and efficiency of hybridization of DNA on to derivatized glass surfaces. This makes use of the synergistic effect of two binding interactions between the nucleic acids and the coating chemicals on the surface of the glass slides. The enhanced binding allows the nucleic acids to be bound tightly and to survive stringency washes. When immobilized,

DNA exhibits a higher propensity for hybridization on the surface than on slides with only one binding chemical. By varying the silane concentrations, we have shown that maximal DNA oligonucleotide binding on glass surfaces occurs when the percentage composition of both of the surface-coating chemicals falls to 0.2%, which is different from that on binding PCR products. This new mixture-combination approach for nucleic-acid binding allows signals from immobilization and hybridization to have higher signal-to-noise ratios than for other silane-coated methods.

Key words: DNA microarray, DNA immobilization efficiency, silane derivatization.

INTRODUCTION

The DNA microarray technique has been used in a wide variety of applications, such as in global gene expression, nucleotide polymorphism detection, disease screening, diagnostics and nucleic acid sequencing [1]. There are two ways to produce surface-immobilized DNA on the solid phase for microarray. One is by direct chemical synthesis on the solid surface by photolithography [2,3] or by phosphite-triester chemistry [4], and the other is by immobilization of pre-fabricated nucleic acids. Photolithography is the less popular method of choice, because it lacks flexibility and also because of the high cost of production. In the direct deposition method, the solid support can be either glass slides or membranes.

Glass slides are more popular than nylon membranes for microarray fabrication, since nylon membranes have several inherent disadvantages, such as high autofluorescence and fragility, whereas glass has a low intrinsic fluorescence and superior optical characteristics for fluorescent scanning. The convenience of printing either PCR products or oligonucleotides without a large length restriction makes it far preferred as the method of choice for microarray fabrication.

Nowadays, a myriad of methods are available for the attachment of nucleic acids on to glass or silicon surfaces. Some of these employ activation of nucleic acids by conjugating an active silyl [5] or thiol moiety [6,7]. Another attachment method involves coating silane with active functional groups on the glass surface. The most economic method, however, is coating with polylysine [8], but unfortunately this suffers from the problem of aging, which affects the consistency of hybridization after storing for several months. Other popular substrates include amine-silane [9] and aldehyde-silane [10], as well as others such as poly-

ethyleneimine polymer [11], *p*-aminophenyl trimethoxysilane/diazotization chemistry [12] and dendrimeric structure [13].

All of the methods mentioned make use of only a single functional group for attachment between nucleic acids and glass surface. In some cases, the effectiveness of immobilization is enhanced by irradiation with UV light to form extra bonds. However, the efficiency of the UV cross-linking is not high, and therefore the nucleic acids on the slide surface cannot survive harsh treatments, such as stripping.

In the present paper, we report a method that enhances the efficiency of binding of nucleic acids on to the glass surface, which provides more than one kind of functional interaction between the nucleic acids and the glass surface. The synergistic effect of epoxy-silane and amine-silane together results in a much higher binding capacity for both PCR products and oligonucleotides, and provides an efficient surface for hybridization. Optimized binding of oligonucleotides on the glass surface occurs at silane concentrations that are different from that of double-stranded PCR products.

EXPERIMENTAL

Nucleic acids employed

Poly(A)_n was bought from MWG-Biotech Inc. (Ebersberg, Germany) and human Cot-1 was from Invitrogen (Carlsbad, CA, U.S.A.). Unmodified, amine-linked or Cy3 or Cy5 (cyanine dyes 3 or 5)-labelled oligonucleotides of different lengths were synthesized by MWG-Biotech Inc. (Singapore). The DNA sequences of the oligonucleotides were all derived from a segment of *Arabidopsis thaliana* ATP sulphurylase precursor (ASA), and were selected by an in-house computer program (U-GET;

Abbreviations used: ASA, *Arabidopsis thaliana* ATP sulphurylase precursor; Cy3/Cy5, cyanine dyes 3 and 5 respectively; ddH₂O, double-distilled water; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; SSC, standard saline citrate.

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The patent on the technology developed in this paper has been filed to the US Patent Office (patent application number 10/226,787) and Taiwan Intellectual Property Office (patent application number 90129226).

U-Vision Biotech, Taiwan) to ensure that they were unique and had a minimal level of secondary structure. PCR products of different sizes were generated with a pair of primers specific for the SK(−) cloning vector, containing DNA fragments of the following genes with *Taq* DNA polymerase (Viogene, Taiwan): a 983-bp-long fragment of the G3PDH (glyceraldehyde-3-phosphate dehydrogenase) gene; a 800 bp *Arabidopsis thaliana* ATP sulphurylase precursor (ASA) gene; a 2322 bp transferrin receptor gene; a 556 bp ribosomal S9 gene; and a 298 bp β -actin gene. Their PCR products were amplified with the following cycling program: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min for 30 cycles, before the reaction was finally stopped at 4 °C and removal from the MWG Primus 96 Thermocycler (Ebersberg, Germany). Amine-linked PCR products were prepared by the same procedure, except that the two primers were amine-modified at the 5' end. The PCR products were purified from the unlabelled nucleotides by using QIAquick nucleotide removal kit (Qiagen, Hilden, Germany). Amine-silane slides were purchased from TeleChem International Inc. (Sunnyvale, CA, U.S.A.) and Corning Inc. (Corning, NY, U.S.A.). Three-dimensional polymer microarray slides were from SurModics (Eden Prairie, MN, U.S.A.).

Fabrication of microarray slides

The coating solution was made by stirring a mixture of between 0.1 and 1 % (3-glycidyloxypropyl)trimethoxysilane (United Chemicals Technologies, Bristol, PA, U.S.A.) with between 0.1 and 1 % of (*N,N*-diethyl-3-aminopropyl)trimethoxysilane (United Chemicals Technologies) and tetramethylammonium hydroxide (Lancaster, U.K.) at 4 °C for 30 min, and the concentrations of the two silanes were varied according to different experiments. Glass slides were cleaned by ultrasonication in double-distilled water (ddH₂O) for 30 min, soaked in 10 % (w/v) NaOH for 60 min, rinsed with running water for 5 min, and then dried by spinning at 50 g for 5 min. The slides were then immersed in the coating solution for 5 min before being spun dry in a spin coater, and then were put in an oven at 80 °C for 20 h.

Immobilization of nucleic acids on slide surface

Printing Cy3- or Cy5-labelled or unlabelled PCR products or oligonucleotides was performed by using a Cartesian PixSys 5500 Arrayer (Cartesian Technologies Inc., Irvine, CA, U.S.A.). The concentrations of the printed nucleic acids were from 0.2 to 0.5 $\mu\text{g}/\mu\text{l}$ for PCR products, and from 2 to 20 μM for oligonucleotides. Both PCR products and oligonucleotides were dissolved in a 50 % (v/v) DMSO solution, before spotting on to the slide surface. The nucleic acids were then immobilized by placing in a humid chamber, which was made by putting a small volume of saturated sodium chloride solution on the bottom of a beaker (at 42 °C for either 2 h for PCR products or 20 h for oligonucleotides). The slides were washed with vigorous shaking in 0.1 % (w/v) SDS for 1 min, and then washed further with gentle shaking for 5 min. The slides were then boiled for 2 min, rinsed with ddH₂O and dried by spinning at 50 g for 5 min. No capping is needed for the slides, because the amine employed is a tertiary amine that cannot react with succinic anhydride.

Preparation of RNA transcripts

Total RNA was isolated using TRIZOL[®] reagent (Invitrogen, Carlsbad, CA, U.S.A.), and mRNA was purified using the Qiagen mRNA Midi Kit (Qiagen). mRNA was reverse-transcribed into cDNA using 13 μM random hexamer with 1 $\times$ first-strand buffer

[10 mM dithiothreitol/500 μM dNTPs (dATP, dCTP and dGTP)/200 μM dTTP/0.2 unit of RNasin (Invitrogen)/13 units of Super-script II (Invitrogen)]. Either Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) (100 μM each) was incorporated into cDNA synthesis during reverse transcription. The reaction mixture was heated to 70 °C for 10 min, and then placed at room temperature for 10 min to allow primer annealing. After adding Cy3- or Cy5-dUTP and the enzyme, cDNA synthesis was allowed to proceed at 42 °C for 90 min, followed by the addition of 30 mM sodium hydroxide at 70 °C for 15 min to hydrolyse the RNA in the mixture. The alkaline was neutralized by adding 30 mM HCl. Unincorporated nucleotides were removed by using the Qiagen QIAquick[™] Nucleotide Removal Kit, and the DNA was then concentrated by using Microcon YM-30 (Millipore, Bedford, MA, U.S.A.). Alternatively, the mixture was purified by precipitating the solution with 70 % (v/v) ethanol for 1 h.

Sample preparation, hybridization, scanning and analysis

After the labelled cDNA derived from 1 or 2 μg of mRNA was mixed with 20 μg of poly(dA)_n and 20 μg of human Cot-1 DNA, the mixture was then concentrated with a Microcon YM-30 Concentrator (Millipore). Equal volumes of 2 $\times$ hybridization buffer containing 50 % formamide, 10 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.2 % (w/v) SDS were added, before heating at 95 °C for 3 min to denature the probe. The cDNA mixture was applied on to the arrays, and covered with a glass coverslip. The arrays were then placed in a hybridization chamber, which was made by adding some wet towels on to the bottom of a slide box to prevent the slides from drying during hybridization. The chamber was put inside a hybridization oven at 42 °C for 16 h, when the hybridization buffer described above was used, or alternatively for 1 h when using EasyHyb Hybridization buffer (U-Vision Biotech Inc., Taiwan). The results obtaining from using either solution were very similar, except that the times for hybridization were different. After hybridization, the arrays were washed sequentially with 2 $\times$ SSC/0.1 % SDS at 42 °C for 5 min, 0.1 $\times$ SSC/0.1 % SDS at room temperature for 10 min, and then 0.1 $\times$ SSC for 5 min. The arrays were rinsed with ddH₂O and dried by centrifugation at 50 g for 5 min. For all the experiments described in this paper, the average signal values were taken from the spots of three slides processed in parallel. The fluorescence intensities were detected with a ScanArray 3000 unit (Packard, Downers Grove, IL, U.S.A.), and then quantified with Imagen 4.0 (Biodiscovery, Inc., Marina del Rey, CA, U.S.A.). The same power and PMT (photomultiplier tube) setting values were used for the same set of experiments.

Stripping the arrays

Fluorescent signals on microarrays were removed by boiling in a stripping solution containing 0.05 $\times$ SSC, 10 mM EDTA, pH 8, and 0.1 % SDS for 10 min. The slides were rinsed with 0.01 $\times$ SSC at room temperature, before scanning for the signal. The stripped slides were hybridized as for the first time with the same labelled cDNA mixture, as described above. The stripping and hybridization steps were repeated two more times, and the signals on the slides were collected after each round of hybridization and stripping.

RESULTS

Synergistic effect of two different silanes

Immobilization of nucleic acids on the glass surface for microarray experiments usually involves derivatization of the

Figure 1 Synergistic effect of two silanes on immobilization and hybridization of unmodified or amine-linked DNA

(A) The signals of Cy3-labelled G3PDH PCR product (983 bp) remained on slides that were coated with epoxy-silane, amine-silane and (epoxy + amine)-silane after extensive washing. (B) The signals from hybridization of the G3PDH PCR product spotted on slide surface with labelled cDNA of the same gene. In both (A) and (B), '1' represents the signals from the epoxy-silane slide (white bars); '2', signals from the amine-silane slide (dark-grey bars); '3', summation of the signals of epoxy-silane (1) and amine silane (2) slides together (light-grey bars); '4', signals from epoxy + amine silane mixture slides (black bars).

glass surface with silane molecules of different chemical functional groups, such as primary amine-, epoxy- or aldehyde-groups. The ability of each derivatized surface to attach nucleic acids varies, depending on the functional groups and the attachment condition. We were interested in investigating whether a combination of two different chemicals would give a better substrate for nucleic acid immobilization and hybridization. We derivatized the glass surface either with a single silane, or with a mixture of two silanes among three types: (N,N-diethyl-3-aminopropyl)trimethoxysilane (a tertiary amine-silane), (3-glycidyloxypropyl)trimethoxysilane (an epoxy-silane) and aldehyde-silane. A tertiary amine was used instead of a primary one because the tertiary amine group retains a permanent positive charge for attracting the negatively charged DNA backbone, and also because it is essentially non-reactive to many chemicals, including the epoxy-silane, when they are mixed together during coating.

Fluorescent dye-labelled PCR products were prepared and dissolved in many different buffers, including 3 × SSC, 50% DMSO, 50 mM sodium bicarbonate buffer at pH 10 and sodium phosphate buffer, pH 7. They then were spotted and immobilized on different slide surfaces, and the slides were then treated with all the washing steps that were used in hybridization (see the Experimental section). This treatment was to test the percentage of spotted DNA that remained on the slide surface. After spotting the DNA on the slides, we did not block the unreacted amine, because tertiary amine is not reactive towards succinic anhydride and the blocking step did not change the background signals at all (results not shown). The fluorescent signals that remained on the slide surface after spotting and treatment with various washing buffers were quantified. The amount or percentage of PCR products that remained on the slide surface varied greatly, depending on the type of silane (results not shown). In the present paper, we have described only the combinatory effect of epoxy and tertiary amine-silanes on the immobilization efficiency of nucleic acids on a glass surface.

A Cy3-labelled PCR product of the *G3PDH* gene was spotted on to the surface of glass slides derivatized with epoxy-silane, amine-silane, and epoxy- and amine-silane together (epoxy + amine) respectively. The slides were washed to remove excess unbound DNA. The amounts of labelled DNA that remained on these slides were measured with a confocal scanner. As shown in

Figure 1(A), the amount of DNA immobilized on epoxy-silane slides (white bars) was much lower than that on amine slides (dark-grey bars), whereas the DNA retained on the epoxy + amine mixture slide (black bars) was twice the level of that of the amine-silane slide (light-grey bars), rather than just the summation of signals of the two slides. The same effect was observed when the spotted PCR products were amino-modified (right-hand bars of Figure 1A), although the effect was less prominent. These results suggest that the synergistic effect occurs only when both the epoxy group and the amine group are present together on the slide surface.

We investigated further whether the synergistic effect could also lead to a higher hybridization signal. The G3PDH DNA was spotted and hybridized with a Cy3-labelled PCR probe (Figure 1B). A very similar result was obtained from the hybridization experiment. The hybridization signals from the epoxy + amine mixture slides (black bars) were higher than those from the amine-silane (dark-grey bars) or epoxy-silane (white bars) slides. These observations indicate that the additional immobilized DNA on the (epoxy + amine) mixture slide is readily available for hybridization.

Optimization of the concentrations of the two chemicals

We then sought to determine the optimal concentration (percentage composition) of the two silanes that yielded the highest signal of hybridization from immobilized PCR product by varying the concentration of one silane, while keeping the other one constant. As shown in Figure 2(A), increasing the concentration of amine-silane alone enhanced the signal of hybridization very sharply, which reached a maximum at a concentration of amine-silane of approx. 1.0–2.0%, before falling in a linear manner.

On the other hand, when increasing the concentration of epoxy-silane while keeping amine-silane at 1%, the signals of hybridization also increased sharply, but reached a plateau at [epoxy-silane] 0.5% (Figure 2B). Unlike amine-silane, when the epoxy-silane concentration was > 1%, the hybridization signals did not change (Figure 2B). Therefore, a mixture of amine-silane:epoxy-silane (each at a concentration of 1%) was used to derivatize the glass surface for the immobilization of PCR products. These two sets of experiments demonstrated that the ratio between the

Figure 2 Optimization of hybridization of G3PDH PCR product (983 bp) by changing the concentration of one silane at a time on the mixture-silane slide

(A) Effect of variation of amine-silane concentration, while keeping the concentration of epoxy-silane at 1%. (B) Effect of variation of epoxy-silane concentration, while the concentration of amine-silane was kept at 1%.

two types of silane is important in creating the synergistic effect that helps to immobilize DNA on the slide surface.

Comparison of immobilization methods

We tried many different methods to increase the amount of DNA spotted on to the derivatized surface, and we found that UV-irradiation was almost ineffective in enhancing the immobilization of PCR products (Figure 3D), with only 50-mer and 70-mer oligonucleotides able to be cross-linked to a greater extent at higher UV dosages (Figure 3C). We found that an efficient method for enhancing immobilization was incubating the spotted slides in a humid chamber for different lengths of time at 42 °C. As shown in Figure 3(A), the amount of oligonucleotides immobilized on the slide surface increased with the length of time of incubation under the humid conditions (filled circles), but the increase was not so high when the spotted slides were placed outside the humid chamber with a humidity of approx. 60% (open circles). On the other hand, humidity had only a slight effect on the immobilization of PCR products of different lengths (between 983 and 2300 bp) compared with that on oligonucleotides (Figure 3B). By comparing the two methods, the immobilization method at 42 °C in a humid chamber was the preferred one, because this was both more highly effective and also more convenient, without requiring a UV cross-linker (Figure 3E).

Tight binding of nucleic acids on the slide surface

The experiments described above suggested that the nucleic acids could be bound tightly on to the slide surface; therefore we went on to investigate whether the DNA bound on the slide could survive harsh treatments. To determine the amount of DNA retained on the derivatized slide surface after harsh treatment, we immobilized Cy3-labelled G3PDH PCR product on to the slide surface, washed this with 0.1% SDS twice and boiled the slide for 2 min to remove any unbound DNA. A total of 76.5% of the DNA stayed on the surface, compared with that before treatment (Figure 4A). A second identical treatment showed that no further bound DNA was washed away, indicating that the remaining nucleic acids were linked to the slides tightly. This experiment suggested that the slides could be used many times by repeated stripping.

To evaluate the reusability of the slides, we spotted and immobilized the PCR product of the plant ASA gene (800 bp) and a 70-mer oligonucleotide of the same gene on the slide surface. Hybridization signals (denoted in Figure 4B as Hyb1, Hyb2 and Hyb3) were obtained by confocal scanning after hybridization with a Cy3-labelled 70-mer oligonucleotide of the ASA gene (Figure 4B). The hybridization signals were then stripped in a boiling stripping buffer for 10 min, and the slides were scanned again (Figure 4B, 'Strip'). The above procedure was repeated two more times. There was a gradual decrease in the signals (70% to 60% loss on average) after each round of hybridization and stripping. The decrease in the ability of the DNA to be hybridized after stripping may be explained in two possible ways. One possibility is that more bound DNA on the slide surface may be removed by high temperature (98 °C). The proportion of oligonucleotide removed by each round of stripping was more than that of long-PCR product, because oligonucleotide molecules lack the 'spaghetti' effect (a tangling of the long denatured DNA strand enables it to become trapped inside the spot on the slide surface). The second possibility is that the PCR product on the slide surface might be less available, owing to the change in the layer structure of the DNA on the slide surface [14]. The results demonstrated that the slides were reusable, because the DNA retained on the slides was still available for hybridization and the residual background signals after stripping (6–10%) were still well below the coefficient of variation of signals if the slides were used for repeating the similar experiments.

Comparing the binding efficiency with other chemistries

With the optimized protocol, we compared the DNA-immobilization efficiency of the G3PDH PCR product on different derivatized chemistries. The chemistries tested included the one we developed in this paper (amine + epoxy mixture slide), amine alone, poly(L-lysine) and polymeric substrates for comparison. The proportion of DNA retained on different slides varied with the chemistry and concentration of spotted DNA (Figure 5A). With the amine + epoxy-silane slides, the DNA appeared to bind very efficiently, and this was almost independent of the concentration of the DNA spotted (from 0.2 to 1.6 µg/µl). The other types of slide showed different behaviour, depending on the concentration of the DNA. For example, the amine-silane slide could bind most of the DNA when the concentration was low, whereas approximately one-half of the DNA could be washed away when the concentration of the DNA was high. Surprisingly, the results with the polylysine-coated slide were exactly opposite: a higher DNA concentration resulted in more DNA binding. We are not able to provide a universal mechanism that can explain these observations, but believe that these results may be accounted for by the fact that different slide surfaces with different

Figure 3 Effects on hybridization of nucleic acids at high humidity or by UV-irradiation

Signals from hybridization of the nucleic acids with their respectively labelled complementary DNA were measured on the slides that had been treated by either one of the two immobilization methods on the slides. **(A)** Oligonucleotides (70-mer and 30-mer of ASA gene) or **(B)** PCR products (2322 bp from transferrin receptor gene and 983 bp from *G3PDH* gene) were kept either in a humid chamber or in the drawer (humidity at 65 to 70 %) for different lengths of time (from 0 to 24 h). Alternatively, oligonucleotides (10–70-mer) or PCR products (983, 556 and 298 bp) were spotted and irradiated with UV light at the different dosages indicated. **(E)** Direct comparison of the signals from both methods.

Figure 4 Tight immobilization of DNA on the slide, and reusability of the slide

(A) The signals from 30 spots of Cy5-labelled *G3PDH* PCR product (983 bp) immediately after printing on the slide (bar 'S'). The signals from the slides were measured after the slides were washed with 0.1 % SDS twice for 5 min and boiled for 2 min (bar 'W1'); the signals after a further round of the same treatment are shown by bar 'W2'. **(B)** Unlabelled 800 bp PCR product and a 70-mer oligonucleotide were spotted on to the slides and hybridized with a Cy3-labelled complementary 70-mer overnight. The signals were obtained from the hybridization (Hyb1), and after stripping (Strip1). The same procedures were repeated two more times, and the signals after hybridization and after stripping were Hyb2 and Hyb3, and Strip2 and Strip3, respectively.

hydrophobicities would allow different amounts of DNA to be transferred (Figure 5B; see the 'Before wash' data), and that the sizes of the spots are also different. Moreover, the actual amount of DNA bound on the slides ('After wash') and the signal-to-noise ratios were highest on the mixture-chemistry slide, whereas the other slides had lower signals and signal-to-noise ratios (Figure 5B). We conclude that the mixture chemistry provides a better substrate for DNA immobilization and hybridization than other slides.

Optimization of oligonucleotide binding

During the optimization process, we observed that the binding efficiency of the oligonucleotide might also depend on the concentration of silanes on the slide surface (results not shown). To optimize oligonucleotide binding, we varied the concentrations of both the epoxy- and amine-silanes for maximal binding and hybridization of oligonucleotides of different lengths. Strongest hybridization signals were observed for different lengths of

Figure 5 Comparison of immobilization of long PCR products on different slide surfaces

(A) The efficiency of immobilization was calculated as the percentage of the labelled PCR product (983 bp long) that was retained on the slide surface after printing, and treated with the whole hybridization procedure (washing steps), although without hybridizing with a labelled DNA. Different concentrations of the same PCR products (from 0.2 to 1.6 mg/ml) were printed on the same slide surface, and the results were displayed with different colours. 'Amine + Epoxy' represents the mixture slide developed in this paper; 'amine' slide was from TeleChem; the three-dimensional (3-D) polymer was from SurModics; and the poly(L-lysine) slide was made in-house according to the protocol from Patrick Brown's laboratory at Stanford University (see http://cmgm.stanford.edu/pbrown/protocols/1_slides.html). (B) The images and the values of the DNA spots before and after washing. The signal mean and the signal-to-noise ratio are derived from values from 18 spots, and the variations in signals are shown as standard deviations (Signal $\pm$ st dev). Signal-to-noise ratios are calculated by dividing the signal means with the background means. The rainbow-coloured bar represents the signal intensity of the spots from scanning. The red colour represents the highest signal intensity, and the purple colour represents the lowest intensity.

Figure 6 Maximization of hybridization signals from oligonucleotides on the slide surface by dilution of the concentration of both coating silanes on slide surface

(A) The signals of the oligonucleotides of 30 (●), 50 (○) and 70 (▼) nt in length. (B) The signals from slides that were printed with PCR products of different lengths: 298 bp (●), 556 bp (○), 983 bp (▼) and 2232 bp (▽). This experiment was to show the differences in behaviour among PCR products from oligonucleotides on the slide surface with different concentrations of the two silanes.

oligonucleotide (30-, 50- and 70-mers) when the concentration of both epoxy- and amine-silanes was 0.2% (Figure 6A). The 70- and 50-mers were affected more by the concentration than the 30-mer. However, a similar trend in hybridization signals from PCR products on the slide surface was not observed (Figure 6B).

Finally, we carried out a comparative study to find out the differences among other chemistries on the hybridization efficiency of oligonucleotide. As shown in Figure 7, a decrease in silane concentration (the '0.2%' bar) resulted in a 3-fold increase in signal, on comparison with the formula (the '1.0%' bar) that was developed for PCR product. Moreover, the hybridization efficiency of oligonucleotide on this slide was much higher than those of other amine-silane-coated slides (Figure 7, the 'Amine-1' and 'Amine-2' bars). The 0.2%-mixture-silane slide was also

the one with the highest signal-to-noise (S/N) ratio (S/N 164.8), whereas the S/N ratios of other slides were approx. 20–30.

DISCUSSION

From our experiments, we found that the general procedure for making a derivative surface on a glass slide, by using a single silane (i.e. amine-, epoxy- or aldehyde-silane), does not generate the most efficient surface, although, of these, aldehyde-derivatized surfaces have been shown to be the best option [10]. In the present study, we have shown that the combination of tertiary amine and epoxy silanes at a ratio of approx. 1:1 produces the greatest synergistic effect to immobilize DNA, and yields the highest

Figure 7 Comparative study of hybridization capability of oligonucleotides and PCR products on different types of slides

The signals from the oligonucleotide spots after hybridization with their Cy3-labelled complementary oligonucleotides are shown. The insets are the images from the scanning of the slides, and only three represented spots from a total of 15 spots are shown. 'Amine-1' represents the Corning CMT-GAPS™ slide, and 'Amine-2' is the TeleChem SuperAmine™ slide.

hybridization signals. We propose that the interaction between the positively charged tertiary amine of the silane molecule and the negatively charged phosphate backbone of the DNA helps the nucleic acids to be immobilized stably on the slide surface [15], whereas the epoxy group forms a covalent linkage with the primary amine groups on the bases of the DNA. The presence of each interaction simultaneously with the other probably enhances the effect of the other to create the synergistic effect observed.

We employed tertiary amine, rather than primary amine, groups as a modifier for derivatization, because tertiary amines have low reactivity towards the epoxy group upon their mixing together before coating on the slide surface. Moreover, the tertiary amine group provides a permanently positive charge that is not affected even by high pH, as is the primary amine group. Since the pK_a value of primary amine is approx. 10, the alkaline pH that is needed for the epoxy group reaction with the amine on DNA would not neutralize the positive charge on the tertiary amine-silane. We found that the best spotting solution is a solution of 50% DMSO in water, because this was the solution that yielded the best spot morphology and immobilization efficiency (results not shown). We tried using a 50 mM sodium bicarbonate solution at pH 10 to favour the epoxy reaction with the amine group on DNA, but this did not give a better result than the 50% DMSO solution, and it also created bright 'speckles' in the spots. The formation of the speckles may be due to the formation of crystals of sodium bicarbonate after drying.

The comparative study showed that the mixture chemistry provides a higher binding capacity and affinity for DNA than any single silane-coated slides, and the immobilized DNA can survive very harsh washing and stripping treatments. Moreover, we observed that lowering the concentrations of both silanes at the same time can allow much higher efficiency of binding and hybridization of the oligonucleotides on the slide surface. Therefore the resulting slide surface has a very high capacity for oligonucleotide hybridization, and thus high a signal-to-noise ratio, when compared with other coating chemistries.

We believe that the interaction between DNA and the epoxy group is different from that of DNA and the amine group, as explained by the following observations. First, during the whole process of derivatization, the pH of the silane mixture is always

kept neutral in order to protect the epoxy group from hydrolysis. Secondly, the synergistic effect is maximized when the ratio of the two silanes is 1:1 (Figure 1A). Higher amine concentrations exceeding the ratio leads to a fall in DNA immobilization, and this effect is not seen upon changing the epoxy concentration, suggesting that the amine group interacts differently from the epoxy group with the DNA. Thirdly, stripping the slide surface at high temperatures does not lead to a complete loss of spotted DNA, because a proportion of the DNA molecules is bound covalently on the surface with the epoxy-silane (Figure 4). Moreover, the synergistic binding is not likely to be due exclusively to an increase in density of hydrogen bonding on the slide surface [16], because experiments using 10-fold-diluted mixed-silane solution, which results in a much lower density of hydrogen-bonding molecules, leads to the highest immobilization of oligonucleotides and no effect on immobilization of PCR product (Figures 6A and 6B). If epoxy-silane or its hydrolysed product is only providing additional hydrogen bonds to those of the amine groups, an additive, rather than a synergistic, effect should be observed.

In the present study, we have only shown data for the synergistic effect of the mixture chemical coating on a limited number of PCR products and oligonucleotides; however, these observations can be extended to other DNA molecules of different lengths and forms (results not shown). The DNA molecules that we used during the development of the coating solution are of different lengths (10 to 2300 bp) and nucleotide compositions, and all of them showed an enhanced binding on the mixture-coating slide surface. Both ourselves and our slide users from many other parts of the world have tested and compared the mixture slides and other brands of amine or aldehyde slides in a high-density format, and most of these tests showed higher signals of either binding or hybridization. Although what we have discussed here is an interesting finding, we do not know the exact mechanism underlying the synergism. Experiments are being performed to determine the mechanism of the synergism observed.

We thank our colleagues in the Department of Production for providing important technical details during the production period of the slides, as well as other colleagues in the Department of Research and Development for their comments and testing on the slides. We would also like to thank Mark Her for his technical comments during the early phase of the project.

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Received 31 March 2003/12 June 2003; accepted 16 June 2003

Published as BJ Immediate Publication 16 June 2003, DOI 10.1042/BJ20030486