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A Factorial Analysis of Silanization Conditions for the Immobilization of Oligonucleotides on Glass Surfaces

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The modification of glass surfaces with (3-mercaptopropyl)trimethoxysilane and the application of this to DNA chip technology are described. A range of factors influencing the silanization method, and hence the number of surface-bound, chemically active thiol groups, were investigated using a design of experiment approach based on analysis of variance. The number of thiol groups introduced on glass substrates were measured directly using a specific radiolabel, [^{14}C]cysteamine hydrochloride. For liquid-phase silanization, the number of surface-bound thiol groups was found to be dependent on both postsilanization thermal curing and silanization time and relatively independent of silane concentration, reaction temperature, and sample pretreatment. Depending on the conditions used in liquid-phase silanization, (1.3−9.0) $\times$ 10^{12} thiol groups/cm^2 on the glass samples were bound. The reliability and repeatability of liquid- and vacuum-phase silanization were also investigated. Eighteen-base oligonucleotide probes were covalently attached to the modified surfaces via a 3′-amino modification on the DNA and subsequent reaction with the cross-linking reagent *N*-(γ-maleimidobutyryloxy) succinimide ester (GMBS). The resulting probe levels were determined and found to be stoichiometric with that of the introduced thiol groups. These results demonstrate that silanization of glass surfaces under specific conditions, prior to probe attachment, is of great importance in the development of DNA chips that use the simple concept of the covalent attachment of presynthesized oligonucleotides to silicon oxide surfaces.

The development of high-density, oligonucleotide arrays or "DNA chips" has revolutionized the fields of genomics and bioinformatics.[1,2] Oligonucleotide arrays can be fabricated by automated spotting and subsequent immobilization of oligonucleotides on to a surface such as glass, nylon, or silicon substrates. Hybridization of complementary targets to these arrays allows the deduction of information such as DNA sequences,[3] drug treatment strategies via differential gene expression monitoring,[4] screening for genetic polymorphisms,[5,6] and evolutionary sequence comparisons.[7] The great advantages of DNA chip-based analyses of genetic material are well known, namely, high-throughput, automation, reproducibility, and speed.

Two main strategies have been developed for the construction of high-density oligonucleotide arrays. One involves the in situ, spatially addressable parallel synthesis of oligonucleotide probes by photochemistries.[8] With these methods, arrays of up to 96 000 different oligonucleotides have been regularly synthesized[6] and used to screen for genetic polymorphisms in the coding region of the hereditary breast and ovarian cancer gene, BRCA 1. The major disadvantages of this method are the high cost associated the design of the photolithography masks necessary for the synthesis of the desired high-density oligonucleotide array and the limited lengths of the synthesized oligonucleotides.

The second method involves the immobilization of presynthesized oligonucleotides or PCR products and has been used for applications such as specific diagnostic tests and drug discovery programs. In this approach, presynthesized oligonucleotides are patterned onto a chemically active surface, using spotting or printing technologies, and immobilized through introduction of functional groups on either the 5′- or 3′-oligonucleotide terminus.

Covalent immobilization methods require chemical modification of the chip surface, so that functionally inert silanols (Si−OH) of a glass surface are modified to possess either nucleophilic or electrophilic functionalities that react with the modified oligonucleotides. It has therefore been necessary to investigate a range of surface chemistries for the attachment of oligonucleotides to inert surfaces, such as glass, commonly used in DNA chips. While strategies such as avidin-modified surfaces with biotinylated oligonucleotide probes have been investigated,[9] most approaches

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favor a covalent attachment of the oligonucleotide to a modified glass (or other substrate) chip.

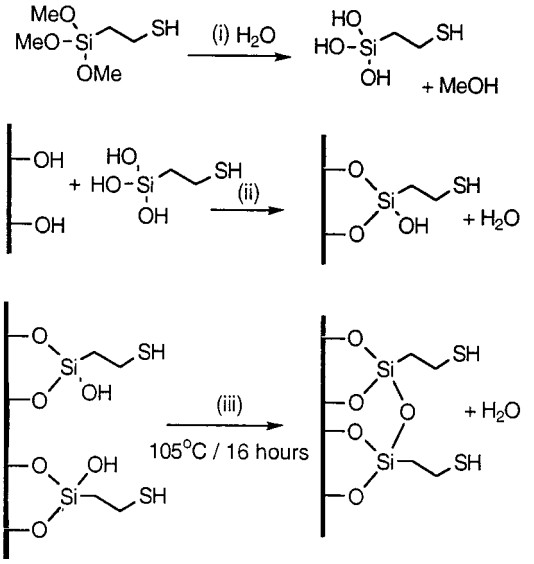
A suitable method needs to efficiently and covalently attach a uniform probe density across the chip surface and not interfere with the highly specific target to probe hybridizations on the immobilized oligonucleotide. It should also be an attachment method that is easily transferable from the laboratory to mass production scale. It should also be reliable, repeatable, and capable of withstanding the conditions employed during blocking and hybridization. If a two-stage probe immobilization method (for example, modification of the surface followed by cross-linking of the probes) is to be used, both stages in the process should be optimized, in terms of a hybridization signal proportional to concentration of target DNA.

To date, the main method for the attachment of biological moieties, such as DNA and proteins, to surfaces, such as those used in optical and electronic biosensors, has involved reaction with organofunctional silanes followed by the covalent attachment of the biological molecule to the newly introduced functional group on the surface.[10−12] Examples of organofunctional silanes used this way include (3-glycidyloxypropyl)trimethoxysilane (3-GPS),[13,14] (3-aminopropyl)triethoxysilane,[15] aminophenyltrimethoxysilane,[16] (3-mercaptopropyl)trimethoxysilane (3-MPTS),[17] and haloacetamidosilanes.[18] All of these have successfully employed modified glass surfaces to immobilize oligonucleotides via various cross-linking reagents.

A range of procedures have been described for the silanization of glass, including elevated[19] and room-temperature organic phase,[17,20] aqueous phase,[21,22] vapor phase, and chemical vapor deposition.[23,24]

The silanization of surfaces has been extensively reviewed in the literature.[19,25−27] In theory, the silanization of surfaces such as glass with mono-, di-, or trialkoxysilanes is relatively simple; hydrolysis of the alkoxy groups yields hydroxyl groups that can covalently interact with the silanol surface, the number of bonding interactions with the surface being the same as the number of hydrolyzable alkoxysilanes. The silane can be cleaved and the silanol surface regenerated by the action of strong base on the generated hydroxyl linkages between silane and surface. However, in reality the situation is more complicated and the exact modification method is still not fully understood.

Scheme 1. Silanization of Glass with 3-MPTS[a]



[a] in (i), the reactive groups of physisorbed silane are hydrolyzed by the surface water on a hydrated silanol (glass) surface, followed by condensation (ii), which leaves the silane covalently bound to the oxide surface, and (iii) thermal curing of the film, which, in cross-linking the free silanol groups, reduces the effect of hydrolysis of one or more of the siloxane linkages to the surface.

It is thought that the initial step is indeed the rapid hydrolysis of the alkoxy groups to liberate silanols and release alcohols (Scheme 1i).[28] The silanol groups then condense with the surface residues to form siloxane linkages. In the case of trialkoxysilanes the presence of three silanol residues in the hydrolysis product can lead to the possibility of multiple surface attachments. The work of Kallury et al. with (3-aminopropyl)triethoxysilane (3-APTES)[19] has shown that providing the number of attachment sites on the surface is not limiting, the preferred conformation of the reaction product of monomeric 3-APTES on a glass or metal oxide has two sites of attachment and the third silanol remains free (Scheme 1ii). This leads to two possible effects. First, with 3-APTES, the activity of the amine group is considerably lowered due to internal self-cyclization and ionization produced by intramolecular hydrogen bonds.[29] This lowered reactivity occurs even after thermal curing, which cross-links the remaining silanol groups (Scheme 1iii). The second effect is common to all trialkoxysilanes, namely, that polymerization can occur at the free silanol group on the surface or in solution prior to condensation

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Table 1. A Range of the Different Silanization Conditions for Glass Substrates Using Trialkoxysilane Reagents[a]

silane	substrate	conditions	thermal curing	ref
3-APTES	zinc oxide	aqueous	no	22
3-APTES	glass			15, 42
				21
3-GPS	CPG[b]/Ballotinibeads	organic phase, catalytic water	no	13
	glass			5
3-APTES	glass		yes	17
3-MPTS	microparticles		yes	10, 20
3-MPTS			yes	
3-MPTS	microparticles	anhydrous organic phase	yes	20
3-APTES	silicon oxide		yes/no	21
3-MPTS	glass	(chemical) vapor deposition	yes	36, 39

[a] The range of different conditions reported has been placed in the four general categories listed, namely: silanization in a predominantly aqueous phase, silanization in an organic phase with catalytic, i.e., ⩽5% v/v H_2O, anhydrous organic phase, and other methods such as chemical vapor deposition and vapor-phase silanization. [b] Controlled pore glass.

with the solid substrate. This leads to a highly polymeric and heterogeneous surface, a potential disadvantage when preparing homogeneous surfaces for DNA chips.

A number of strategies have been developed to limit the formation of heterogeneous polymeric layers relative to homogeneous monolayers. These include limiting the concentration of alkoxysilane prior to surface modification by performing silanization in the vapor phase,[26,30,31] the use of anhydrous conditions, postsilanization curing, and the use of monoalkoxysilanes. However, monoalkoxysilanes are readily cleaved from modified surfaces due to rapid hydrolysis.[32] Dialkoxysilanes have been shown to possess the disadvantages of both, i.e., the polymerization associated with trialkoxysilanes and the instability to hydrolysis of monoalkoxysilanes. Therefore, despite the problems of heterogeneity, trialkoxysilanes are the most extensively used silanization reagents. While postsilanization curing has been shown to limit the hydrolysis of silane films[33,34] by cross-linking of the free silanols, these silanol groups are also free to polymerize and hence form heterogeneous silane layers, unless conditions favoring curing and limiting polymerization are rigorously enforced. The situation is further complicated by the range of silanization conditions reported in the literature for the production of silane films on glass surfaces with trialkoxysilane reagents. Some of the differing silanization conditions reported are listed in Table 1. However, it should be noted that many of the examples cited make no claims as to the natures of the silane layers deposited, i.e., monolayers, polymers, and whether heterogeneous or homogeneous.

While many different silanization conditions have been described for the modification of surfaces, there has been little published with regard to the reliability and repeatability of silanization conditions or how this might also affect oligonucleotide probe immobilization levels.

Most methods of examining silanized surfaces such as X-ray photoelectron spectroscopy (XPS)[19,35] and microscopic methods such as atomic force microscopy (AFM)[21,22,25,35] and infrared spectroscopy (FT-IR)[22,29] provide a qualitative rather than quantitative assessment of functional groups on modified surfaces. To provide a quantitative assessment of functional group surface coverage, various labeling strategies have been reported. For example, the amino group of 3-APTES has been both radiolabeled with [^{14}C]acetic anhydride and fluorescently labeled with fluorescein isothiocyanate (FITC) as a means of determining active-surface amine concentrations.[20] Once qualitative and quantitative measurements of surface coverage have been made, conclusions can be made regarding the surface chemistry, functional group distribution, and reliability and repeatability of the modification chemistry.

The aim of the present work was to determine which experimental factors influenced the silanization of glass substrates, inferred from measurement of the number of functional thiol groups present on the surface following silanization with 3-MPTS. An experimental design procedure, utilizing the analysis of variance (ANOVA), was used to identify the most significant experimental factors in the silanization loading on glass substrates.For this purpose, glass microscope slide covers were silanized under various conditions with 3-MPTS, and the number of thiol functional groups measured using a thiol-specific radioactive label, [^{14}C]cysteamine hydrochloride (Scheme 2). The number of introduced thiol groups was related to the number of silane molecules per unit area and also to the number of sites that could be subsequently coupled to amino-modified oligonucleotide probes with a heterobifunctional cross-linking reagent. To determine which experimental variables (silane concentration, temperature, sample pretreatment, and postsilanization thermal curing) were critical to determining the functional group density on the silanized surfaces, a factorial experimental design was employed.

An understanding of the factors influencing silanization of glass and other surfaces is of great interest in the development of DNA and protein arrays, in offering the potential for controlled surface conditions such as functional group density.

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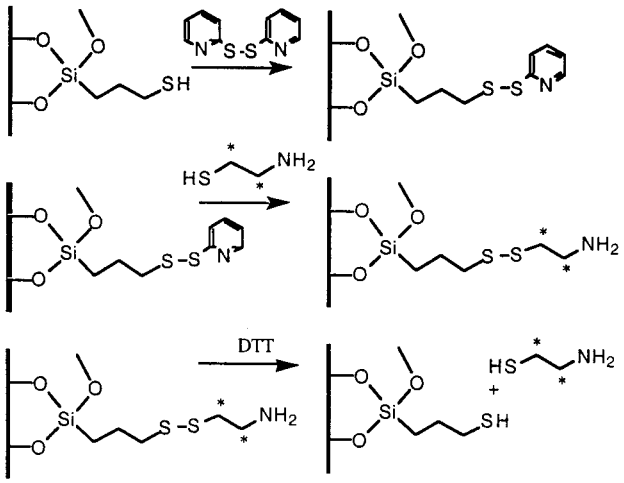
Scheme 2. Method for the Determination of the Number of Active, Surface Cysteines[a]



[a]The modified surface is incubated in dithiobispyridine to activate the surface, followed by [^{14}C]cysteamine hydrochloride and, finally, DTT to selectively release the radioactive probe into solution where it can be measured by liquid scintillation counting.

EXPERIMENTAL SECTION

Materials. Analar grade toluene and ethanol were purchased from BDH (Leicestershire, U.K.) and the ethanol used as such. Toluene was dried using 4-Å molecular sieves. Deionized water was used in all experiments. 3-Mercaptotriethoxysilane was purchased from Fluorochem, (Derbyshire, U.K.) and once opened was stored in a desiccator. Samples not used within 2 months were discarded. Glass cover slips were used as substrates. [^{14}C]Cysteamine hydrochloride was from American Radiochemicals, (St. Louis, MO), [^{33}P]dATP was from AP Biotech (Bucks., U.K.), and Optima Gold was obtained from Canberra Packard (Bucks., U.K.). Care should be taken to ensure sufficient eye protection when using 3-MPTS and concentrated acidic and basic solutions.

Preparation of Glass Substrates. Prior to silanization, the glass substrates were precleaned by treatment in 30% v/v NH_3/30% v/v H_2O_2/water (1:1:5), followed by Piranha solution, HCl/30% v/v H_2O_2/water (1:1:5) at 85 °C for 15 min, followed by extensive rinsing with water, and were stored in 25% ethanol until required.

Alternatively, glass substrates were cleaned by sonication in 1 M HNO_3, then water, and then ethanol for 15 min each. Cleaned glass substrates were stored as above for not more than 20 min prior to silanization. Immediately prior to use, the slides were blow-dried in a stream of dry nitrogen.

Liquid-Phase Silanization. A 20-mL aliquot of 3-MPTS in toluene solution (either 2.5 or 5% v/v) was added to a 50-mL clean and dry glass beaker. A single glass substrate was added to each beaker and the vessel sealed. The samples were incubated at the desired temperature (20 or 37 °C), with gentle agitation of the solution for the necessary time (1 or 4 h). The glass samples were removed and rinsed repeatedly with toluene, ethanol/toluene (1:1), and absolute ethanol. Where appropriate, the samples were thermally cured in an oven at 100 °C for 16 h prior to storage. The samples were then stored in methanol at 4 °C until required. Samples analysis commenced within 24 h of silanization and was completed within 1 week.

Vacuum-Phase Silanization. Cleaned glass substrates were placed in a five-necked glass vessel suitable for reduced-pressure reactions. A thermal probe was fitted to one neck, a pressure gauge to another, and a third was fitted with a Rotaflow tap, which was the point of connection of the vessel to the vacuum line. The remaining vessel necks were sealed with Suba-Seals. The system was evacuated and the temperature of the vessel raised to 120 °C at which point the Rotaflow tap was closed to isolate the vessel from the vacuum line. A 250-μL sample of 3-MPTS was injected into the reaction vessel through one of the Suba-Seals. After 30 min, the Rotaflow tap was reopened and the sample maintained under a positive vacuum for a further 8 h. Samples were removed from the vessel and treated in the manner described above.

Determination of Thiol Group Surface Coverage. All steps were carried out at room temperature in 10 mM sodium phosphate buffer, pH 7.4.[36] Silanized samples were incubated in 30 μM dithiobispyridine for 6 h, after which they were extensively rinsed. The samples were then incubated in a 5−10 μM [^{14}C]cysteamine hydrochloride solution for 24 h and rinsed extensively. Each substrate was incubated in 1.5−2 mL of 30 μM DTT for 12 h. The DTT solution was then added to 10 mL of Ultima Gold scintillation fluid and the number of disintegrations per minute recorded using a Beckman LS 1701 scintillation counter.

Statistical Analyses. A full two-level factorial ANOVA was used to assess the statistical significance of the measured thiol group surface density, using the software package Design-Ease v3.0 (Stat-Ease Inc.). The time required to carry out all the silanization reactions was 4 days; therefore, a blocking design was employed to account for day-to-day variation.

Attachment of 3′-Amino-Modified Probes to the Silanized Glass. Oligonucleotide probes were synthesized by standard phosphoramidite methods by Cruachem Ltd. (Glasgow, U.K.). An amine was attached to the 3′-terminus via a 7-carbon alkyl spacer. Prior to immobilization probes were 5′-radiolabeled with [^{33}P[-γ-dATP according to published methods.[37] A 20-fold excess of *N*-(γ-maleimidobutyryloxy) succinimide ester (GBMS) in DMSO was added to the radiolabeled probe solution (56 μM probe in 50 mM MOPS, pH 7.0) such that DMSO was not greater than 10% v/v, and the mixture incubated at 4 °C for 2 h. The modified probe was purified on a SR100 microspin column (AP Biotech, Amersham, U.K.) and the concentration adjusted to 20 μM, prior to dotting on the silanized glass. The dotted glass was incubated at 4 °C in a water vapor-saturated chamber for 16 h, after which it was rinsed repeatedly with water and air-dried.

Hybridization Conditions. The probe-modified samples were rinsed with 40 mL of water and dried with a paper towel. Each sample was immersed in 5× Denhardt's solution for 15 min at 20 °C. The sample was then rinsed with water, dried, and placed in a clean Petri dish. The sample was covered with the desired target ssDNA solution (20−100 nM) and incubated at 4 °C for 2.5 h. The chip was removed from the target solution, dried, and incubated at 20 °C for 15 min in 2× SSPE (17.5 g of NaCl, 2.8 g of $NaH_2PO_4 \cdot H_2O$, and 0.74 g of EDTA per liter), 0.1% SDS. The chips were removed from solution, air-dried, and either phos-

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phorimaged or treated with 1 M NaOH to release the probe into solution for scintillation counting.

Analysis of Immobilized Probe Levels. (1) Phosphorimaging. Samples were exposed to a Cyclone Storage Phosphor Screen for 15 min and developed using a Packard Cyclone Storage Phosphor System. All image analyses were conducted using OptiQuant software.

(2) Surface Regeneration. Samples were incubated in 1 M NaOH for 16 h, and the supernatant solution was subsequently analyzed by liquid scintillation counting.

EXPERIMENTAL DESIGN

A factorial design of the liquid-phase silanization conditions of glass microscope cover slips was used to elucidate the main factors that determined silane density. Silanization of glass by a chemical vapor deposition method was also investigated.

The experiments necessary for a two-level full factorial design of free thiol group levels under liquid-phase silanization conditions were established using the design of experiments software, Design-Ease. Four different variables at high and low factor levels were chosen, namely: temperature (37 and 20 °C), reaction time (4 and 1 h), silane concentration (5 and 2.5% v/v), and postsilanization thermal curing (16 h at 100 °C, "cured"; 16 h at 20 °C, "uncured"). These variables were selected based on previously published methods (Table 1). Full factorial design with 4 variables at 2 different levels, gave a total of 16 (2^4) different silanization procedures. Moreover, each set of conditions was performed in triplicate, giving a total of 48 different individual silanization reactions to be analyzed. This allowed an assessment of the between-run precision (reproducibility) of the different procedures to be analyzed. Therefore, in total 48 ($2^4 \times 3$) separate liquid-phase silanizations were performed to give a full factorial analysis. The experiments were performed in blocks of 12 on any single day, selected from a randomized list of the 48 experiments. By taking account of this "blocking factor" in the design, effects of day-to-day variation were accounted for in the final analysis.

The within-run reproducibility of silanization was addressed through the analysis of a single set of conditions using 10 cover slips.

RESULTS

Sample Pretreatment. In the literature, many different sample pretreatments have been shown to influence silane loading and uniformity.[38] The aim of this study was not to systematically explore the effects of different sample pretreatments, but rather to elucidate the critical silanization reaction conditions. A preliminary study of two different methods for the pretreatment of glass samples prior to silanization was made. The first method involved incubating the samples sequentially in a hot alkaline and then a hot Piranha solution. At this stage, the silanization conditions had not been optimized and were an incubation in 2.5% v/v MPTS in toluene at 20 °C for 1 h, followed by postsilanization thermal curing for 16 h at 100 °C. The number of thiol groups per centimeter squared were then determined. Analysis showed that there was no significant difference in the mean thiol group density of the two treatment methods ($n = 5$ for both, $P < 0.05$).

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Table 2. The Different Combinations of Experimental Factors in the Liquid-Phase Silanization of Glass

silanization conditions[a]	mean no. of thiols groups $\times 10^{-12}$ cm^2 ($n = 3$)
37 °C/1 h/NC/5%	2.58 ± 0.94
20 °C/4 h/NC/2.5%	3.30 ± 0.84
37 °C/1 h/C/2.5%	2.67 ± 0.10
20 °C/4 h/C/5%	4.48 ± 1.71
20 °C/1 h/NC/2.5%	1.93 ± 0.52
37 °C/4 h/NC/5%	1.34 ± 0.05
20 °C/1 h/C/5%	2.49 ± 0.27
37 °C/4 h/C/2.5%	2.40 ± 0.47
20 °C/4 h/NC/5%	3.34 ± 0.47
20 °C/4 h/C/2.5%	4.63 ± 2.13
37 °C/1 h/C/5%	3.91 ± 1.47
37 °C/1 h/NC/2.5%	2.88 ± 1.29
20 °C/1 h/C/2.5%	3.76 ± 0.63
37 °C/4 h/C/5%	8.07 ± 0.69
20 °C/1 h/NC/5%	2.61 ± 0.40
37 °C/4 h/NC/2.5%	2.18 ± 0.41

[a]Conditions: temperature (°C), reaction time (h), cure ((NC, noncured; C, thermally cured following silanization)), and MPTS (%) v/v in toluene.

Therefore, when we carried out the factorial analysis, the cleaning method was not included as one of the factors.

Analysis of Liquid-Phase Silanization Conditions. A detailed analysis of the results of the 48 separate silanization reactions revealed those experimental variables that were of the greatest significance. The results of the experiment are listed in Table 2. The mean thiol group density on the glass ranged from $(1.34 \pm 0.05) \times 10^{12}$ to $(8.07 \pm 0.69) \times 10^{12}$ cm^{-2}. The results generated in the 48 separate silanizations were then analyzed to determine their factorial dependence. These results indicated that, of the factors analyzed in liquid-phase silanization, the most important was whether the slides were thermally cured or not. These results indicated that a 16-h curing at 100 °C significantly increased the density of free thiol groups on the surface compared to uncured samples. A less significant factor was the silanization time, where increasing the silanization time lead to an increase in the number of free surface thiol groups. This analysis of effects also showed that there was no significant dependence on silane concentrations or silanization temperature at the levels used.

These results are shown in the effects plot (Figure 1a), where the difference between cured and uncured samples is clearly seen. A half-normal probability plot (Figure 1b) shows a random distribution of responses for the other variables while the residual plot (Figure 1c) reveals no systematic day-to-day variation. In all the different experimental conditions investigated, the relative standard deviation (RSD) for thiol group density varied between 4 and 45%. The high standard deviations in silane loadings in some runs may be a consequence of the small sample size ($n = 3$). Indeed, when the run conditions that gave the highest standard deviation were repeated for a larger sample ($n = 10$), the relative standard deviation decreased from 45 to 22%. No common factors were identified as to what influences repeatability in liquid-phase silanization, whereas the factors of postsilanization curing and reaction time were identified as statistically important ($P < 0.05$, degrees of freedom 3) in determining thiol group level following silanization.

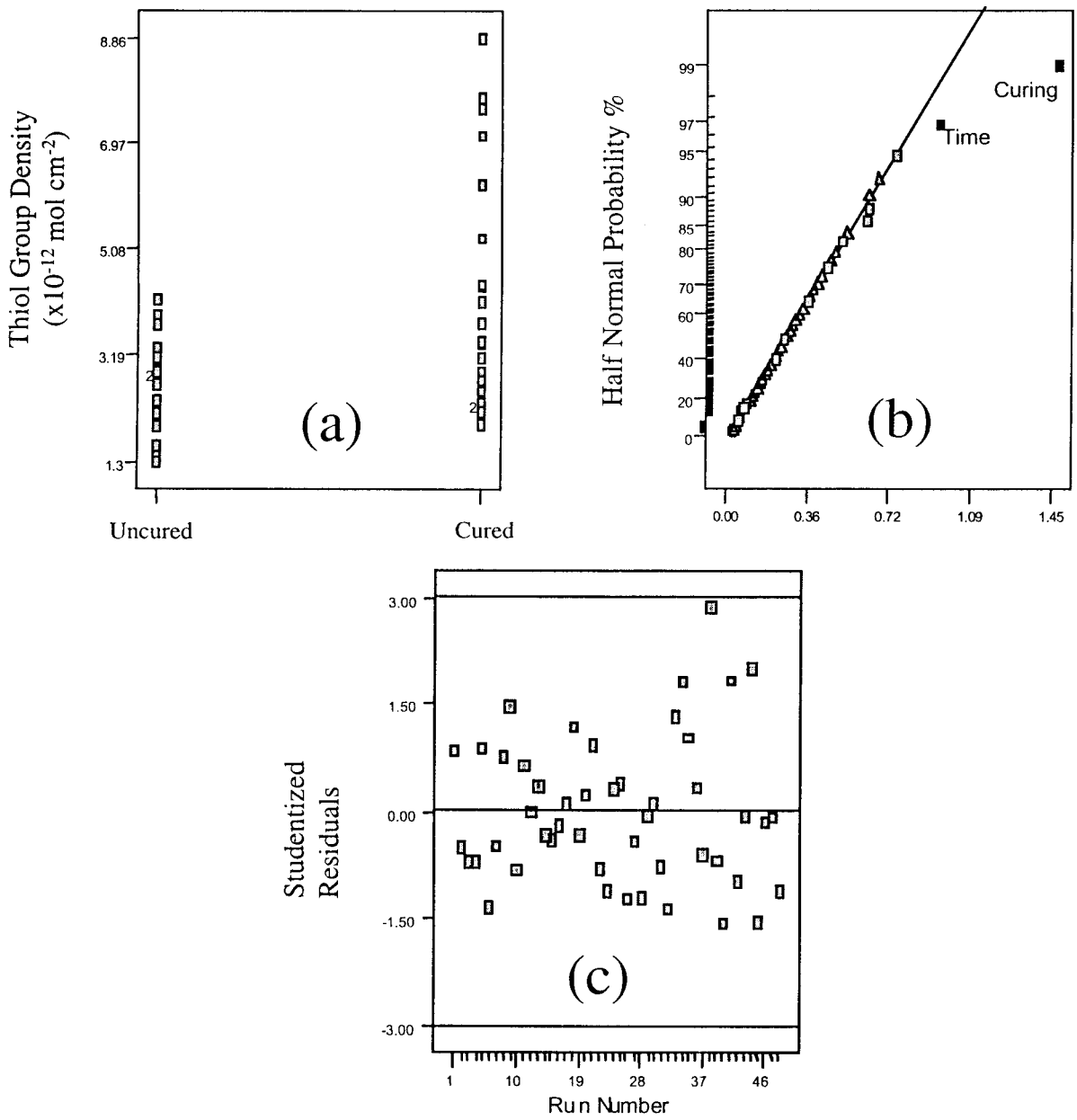

Figure 1. Diagnostic plots for the results of the two-level factorial analysis of conditions for liquid-phase silanization of glass surfaces (a) Effects plot, where each square is the outcome of one run, (b) half-normal probability plot where o represents factorial responses and Δ represents pure error responses. The two most influential factors are labeled, namely, curing and silanization time, and (c) student residuals plot where each square represents a single experiment.

Following the factorial analysis of silanization conditions, one set of experimental conditions was used to investigate within-run variation, i.e., the repeatability. Despite the highest loading being obtained with 5% MPTS, 4-h incubation at 37 °C, and thermal curing, the ANOVA indicated that temperature and silane concentration were not significant factors in silane loading. Therefore, we chose to work at the lower temperature and silane concentration. Ten samples were silanized by incubation for 4 h in 2.5% v/v MPTS in toluene at 20 °C, followed by thermal curing at 100 °C. The number of thiol groups per centimeter squared was then determined for each sample and was found to be $(4.48 \pm 0.98) \times 10^{12}$ for glass ($n = 10$), giving a RSD of 22%. This is essentially the identical ($P < 0.05$) to the mean value of $(4.63 \pm 2.13) \times 10^{12}$ thiol groups/cm^2 ($n = 3$) generated under identical conditions during the ANOVA.

While we acknowledge that thiol group oxidation may occur, the nature of the experiment, i.e., the large number of runs, meant inevitably there was a delay between the curing step and the subsequent quantitation of thiol groups. Even though thiol oxidation may occur, the ANOVA clearly demonstrates (Figure 1a and b) higher thiol loading following thermal curing; i.e., the advantages of a more stable, cross-linked film outweigh the disadvantages of thiol group oxidation either before or during thermal curing.

Vacuum-Phase Silanization Conditions. This method could be used to silanize large numbers of samples relatively quickly. However, it was shown that the numbers of free thiol groups (and hence silane levels) were significantly lower for vacuum than for liquid-phase silanization of glass substrates. It may be expected that if the silane were in a vapor phase, at low pressure (and

concentration) polymer formation would be minimized and a homogeneous deposition of silane would occur on the whole of the sample.[24,30,39] A vacuum-phase silanization of substrates was performed using the conditions described in the Experimental Section. The number of thiol groups per centimeter squared was then determined for each sample and was found to be (1.85 ± 0.13) × 10^{12} for glass (n = 10), giving a RSD of 7%, whereas in liquid-phase silanization under nonoptimal conditions the corresponding values are (4.48 ± 0.98) × 10^{12} (n = 10), giving a RSD of 22%.

Probe Attachment Studies. To establish the relationship between the surface density of thiol groups and the amount of oligonucleotide probe covalently attached to these thiol groups, radiolabeled 18-mer oligonucleotide probes were attached to the silane layer via the heterobifunctional cross-linker GMBS. Glass samples were silanized under liquid-phase conditions (nonoptimized), and the surface thiol group density was determined as 3.8 × 10^{12} cm^{-2}. Oligonucleotide probes were then attached over a defined area of the glass substrates and attached probe levels measured (1) after rinsing of the substrates in water, (2) after incubation with Denhardt's solution, and (3) following hybridization to a complementary single-stranded DNA target.

It was found that after a water rinse (1) probe levels of (8.1 ± 2.6) × 10^{14} cm^{-2} (n = 3) were observed which fell to 3.0 × 10^{12} cm^{-2} (n = 3), i.e., stoichiometric with the number of thiol groups, after incubation with Denhardt's reagent. This corresponds to the very low stringency of the water wash, and therefore, there is probably extensive nonspecific binding of oligonucleotides to the silanized surface. The higher stringency of the Denhardt's reagent breaks up these nonspecific interactions and results in a significant decrease in probe levels. However, if the chip was then used in a hybridization reaction, probe levels continued to fall during the hybridization assay. To examine the stability of the oligonucleotide linkage under conditions necessary to remove bound target and perform subsequent rehybridizations, three hot water washes at 95 °C (n = 3) were performed and a second hybridization assay was performed on the same chip. On subsequent hybridization procedures, probe levels continued to fall to 0.05 × 10^{12} cm^{-2} (n = 1), (corresponding to ~200 dpm on liquid scintillation counting), i.e., some 4% of the total number of thiol groups initially available (Figure 2). While this value is considerably lower than those reported previously, it was found that there was no further loss of oligonucleotide probe from the surface during the hybridization process, indicating that this probe was securely and covalently attached to the silanized chip surface. This decrease in measured probe levels probably represents the loss of material from the surface, possibly due to hydrolysis of siloxane linkages in the hot water washes.

DISCUSSION

The liquid-phase silanization of glass substrates has been investigated with particular attention to the importance of a number of experimental variable factors in determining functional group density. Four factors were investigated for glass substrates, at high and low levels, namely, temperature, silane concentration, reaction time, and postsilanization thermal curing. The levels of silanization of the substrates were determined through quantitation of a thiol group introduced via the silane reagent. It was found that for glass substrates there was no significant difference in thiol group levels with the two different sample pretreatments investigated. An ANOVA was performed for all the different conditions investigated, and it was found that the most important factor in the silanization process was whether the sample was thermally cured following silanization. There was also a lesser dependence on silanization time. For thiol silanes, it has been shown that above 115 °C there is rapid oxidation of the introduced thiol groups, which can be observed by a change in the ratios between reduced and oxidized sulfur on the surface by XPS (J. M. Cooper, personal communication). Hence, it is necessary to limit the curing temperature in the presence of oxygen to between 100 and 105 °C. This does not apply of course to other functionalized silanes, e.g., 3-APTES, where higher curing temperatures may be used.

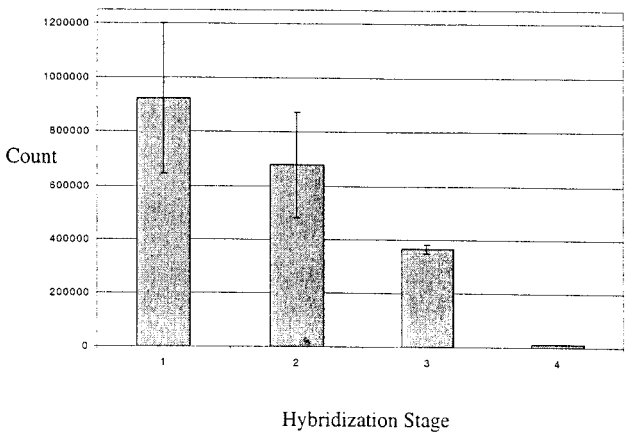

Figure 2. Loss of ^{33}P-labeled oligonucleotide from the surface of a silanized glass chip as measured by phosphorimaging. The probe signals from phosphorimaging correspond to silanized slides with a 20 μM modified-oligonucleotide solution spotted on to the surface following (1) initial water wash (n = 3), (2) incubation with Denhardt's reagent (n = 3), (3) a series of three washes in water at 95 °C (n = 3), and (4) two further hybridization cycles (n = 1).

We conclude that the thermal curing process causes the condensation of unreacted siloxane linkages to produce a cross-linked monolayer of MPTS on the glass surfaces, with a reduced susceptibility to hydrolysis. This thermal curing might also account for some of the lowering of our thiol group levels compared to those reported previously. Of the various factors investigated in the liquid-phase silanization of glass substrates, it was found that greatest silanization levels were achieved using reaction conditions of 37 °C incubation temperature for 4 h, with MPTS at 5% v/v, and postsilanization thermal curing at 100 °C for 16 h. Using these conditions, the mean number of active thiol groups was calculated to be (8.07 ± 0.69) × 10^{12} cm^{-2} on glass substrates.

Calculations based on 3-APMDES have shown that if the silane molecules are packed on the surface to give maximum surface coverage as a monolayer then the number of silane molecules should be ~10^{14} cm^{-2}, assuming that surface sites for silanization are not limiting.[40] This assumes the average density of surface hydroxyl groups on a hydrated silicon oxide surface to be 13.3/nm^2 and the surface area of an immobilized 3-APMDES to be 60

(39) Schmid, E. L.; Vogel, H. *Prog. Biophys. Mol. Biol.* **1996**, *65* (Suppl. 1), 97.

(40) Leonardelli, S.; Facchini, L.; Fretigny, C.; Tougne, P.; P. Legrand, P. *J. Am. Chem. Soc.* **1992**, *114*, 6412−6418.

Å^2. However, in previous work in this laboratory, it was found that silane layers approaching this density were often polymeric and highly heterogeneous.[41] While the numbers of thiol groups, and hence silane molecules, per centimeter squared reported here are much lower, inspection of the surfaces by phosphorimaging of the radiolabeled surface (either by [^{14}C]cysteamine hydrochloride or by [^{33}P]oligonucleotide probes) indicated a relatively homogeneous coverage at the resolution of the phosphorimager (the RSD in intensity across the imaged surface varied between 10 and 15%).

It was found that there were wide differences in the repeatability of liquid-phase silanization of glass, with relative standard deviations ranging from 4 to 45%, under the different silanization conditions. We found that there was no common factor that correlated with repeatability. These results indicate that an important limitation of liquid-phase silanization is that it is difficult to control. The nature of this silanization process probably involves parallel and sequential reactions such as polymer formation in solution or on the surface, hydrolysis of the siloxane linkages, differential surface activation, or incomplete cross-linking of siloxanes on the surface.

To overcome this difficulty, we attempted a chemical vapor deposition of 3-MPTS on glass chips. Using this method, we obtained thiol group densities of $(1.85 \pm 0.13) \times 10^{12}$ thiol groups/cm^2 for glass ($n = 10$). This was comparable to the lower values obtained during liquid-phase silanization (20 °C, 1 h, 2.5% silane, no curing) but with much better within-run variability.

Cross-linking of ^{33}P-labeled oligonucleotides indicated that only some 4% of the thiol groups introduced by liquid-phase silanization were used in the covalent cross-linking of an amine-modified probe to the surface via the heterobifunctional cross-linker GMBS, following water washes at 95 °C. The reason for this low level is unclear but could be due in part to hydrolysis of the siloxane linkages. Indeed, much higher probe levels were found following the removal of nonspecifically bound probe with Denhardt's reagent (3×10^{12} oligonucleotides/cm^2). Total probe levels exceeding the number of free surface thiol groups were encountered prior to the first removal of nonspecifically bound probe on incubation with Denhardt's reagent. On further water washings, immobilized probe levels continued to decline. Despite this, sufficient quantities of probe were stably bound to the glass surface to allow for use in hybridization assays.

In conclusion, we have shown that the main factor in determining silane levels during liquid-phase silanization of glass substrates is the thermal curing of the silanized samples presumably forming stable, cross-linked and condensed silane layer. A lesser dependence was found for the reaction time. This is consistent with the final silane density being determined by the degree of cross-linking of the siloxane groups (and hence presumably their stability toward hydrolysis). Thus, liquid-phase silanization offers a simple means of producing silanized glass substrates; however, the silane density is difficult to control. For a more reproducible surface modification, chemical vapor deposition (or vacuum phase) offers a method that can be performed on a small scale in the laboratory and should be capable of being scaled up for industrial application.

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