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## Strategies for Covalent Attachment of DNA to Beads

**Abstract:** Several covalent attachment chemistries were tested for the immobilization of DNA onto glass beads. The comparison was based on the ability of these chemistries to produce derivatized beads that give good hybridization signals. Cyanuric chloride, isothiocyanate, nitrophenyl chloroformate, and hydrazone chemistries gave us the best (yet comparable) hybridization signals. We further characterized the cyanuric chloride method for the number of attachment sites, number of hybridizable sites, hybridization kinetics, effect of linker length on hybridization intensity and stability of the derivatized beads. © 2004 Wiley Periodicals, Inc. *Biopolymers* 73: 597–605, 2004

**Keywords:** covalent attachment; immobilization; DNA; glass beads; hybridization

### INTRODUCTION

DNA array technology uses microscopic arrays of DNA molecules immobilized on solid supports for biomedical analysis of gene expression, polymorphism or mutation detection, DNA sequencing, and gene discovery.<sup>1,2</sup> There are two distinct approaches to manufacturing microarrays. In one approach, DNA can be synthesized directly on the surface.<sup>3,4</sup> This approach provides wide flexibility in design of custom arrays without preliminary synthesis of modified oligonucleotides. However, in situ synthesis does not allow quality control and purification of individual array features, and incomplete phosphoramidite coupling prevents high yields of full-length oligonucleotide probes, especially for longer oligos.

The second approach uses presynthesized oligonucleotides that are either adsorbed to the array surface or are attached covalently through end-group modifi-

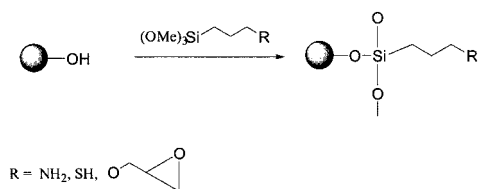
cations that react with functional groups on the solid support.<sup>5–8</sup> A variety of immobilization strategies, including entrapment, adsorption, and chemical binding, have been used to couple nucleic acids to solid supports such as glass, ceramics, silicon, magnetic beads, nylon, polymers, and membranes.<sup>5–12</sup> To maximize stability and to assure that only full-length oligos are attached, we chose to explore methods of covalent attachment through a functional group added during the last step of oligo synthesis.

Illumina's BeadArray™ technology uses silica microspheres (beads) as the array elements.<sup>13</sup> For genotyping and gene expression applications, each microsphere is derivatized with a particular oligonucleotide that acts as a probe for the complementary sequence in an assay solution. In this article, we compare several covalent attachment chemistries based on their ability to produce derivatized beads that give good hybridization signals. For a particular chemistry, we

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**FIGURE 1** General bead silanization scheme.

further characterize the number of attachment sites, the number of hybridizable sites, the hybridization kinetics, the effect of linker length on hybridization intensity, and the stability of derivatized beads.

## MATERIALS AND METHODS

### Chemical Reagents

Silica beads in water (3  $\mu\text{m}$  diameter,  $\sim 5 \times 10^{10}$  beads/g) were obtained from Bangs Laboratories, Inc., Carmel, IN. Silanization reagents, 3-aminopropyltrimethoxysilane, 3-mercaptopropyltrimethoxysilane, and 3-glycidoxypropyltrimethoxysilane were obtained from United Chemical Technologies (UCT), Bristol, PA. Succinimidyl 4-hydrazinonicotinate acetone hydrazone, and 5'-aldehyde modified oligonucleotide 5'-(CHO) TTT GAA AAG CCT ACA CGA CGG CGA A-3' (capture probe) were obtained from Solulink, San Diego, CA. All other oligonucleotides 5'-NH<sub>2</sub>-TTT GAA AAG GGA CGT GCG CTT CGA A-3' (capture probe), 5'-HS-TTT GAA AAG GGA CGT GCG CTT CGA A-3' (capture probe), 5'-FAM-TTT CGC CGT CGT GTA GGC TTT TCA A-3' (target), 5'-FAM-TTT CGA AGC GCA CGT CCC TTT TCA A-3' (target), were obtained from Operon Technologies, Alameda, CA, or synthesized in house using the Oligator™ technology. All oligonucleotides were high performance liquid chromatography (HPLC) purified. All other reagents were obtained from NovaBiochem, Aldrich or Sigma. All solutions were prepared with OmniPur™ (sterile, nuclease free) water from Merck.

### Surface Modifications

**Silanization.** The beads were washed with ethanol prior to derivatization with one of the silane reagents (silanization is shown in Figure 1).

Silica beads (1 g) were washed with 10 mL ethanol (HPLC grade). The beads were then suspended in 10 mL ethanol followed by addition of 50  $\mu\text{L}$  (0.5%) silane reagent, and shaken for 1 h at room temperature. Following silanization, they were washed 5 times with 10 mL ethanol and 3 times with 10 mL ether, dried, and stored at room temperature (RT).

**Ninhydrin Test.** Completion of various reaction steps was monitored (qualitatively) using the ninhydrin test as follows: A small amount of ninhydrin solution (10% by weight ninhydrin in ethanol) was heated to 120°C, a mid-

gen of beads was added to one test tube, and the other was used as reference. When primary amines were present on the beads, ninhydrin solution turned blue; otherwise, the color of the solution remained unchanged.

**Amino (or Thiol) Loading Measurement on Beads.** Silanized beads (1 g) were suspended in methylene chloride (10 mL) followed by addition of 200  $\mu\text{L}$  (1.2 mmol) of diisopropyl ethylamine (DIEA). After a brief sonication, 258 mg (1mmol) 9-fluorenylmethyl chloroformate (Fmoc-Cl) was added into the suspension and the reaction mixture was shaken for 1 h at RT. The beads were washed 5 times with 10 mL methylene chloride and 5 times with 10 mL acetonitrile.

Unreacted amino (or thiol) groups were capped with a mixture of 0.2M acetic anhydride and 0.2M DIEA in methylene chloride (10 mL for 1 g of beads). The suspension was shaken overnight at RT. The beads were washed 5 times with 10 mL methylene chloride and 5 times with 10 mL acetonitrile.

To estimate available amino groups, the Fmoc protecting group was removed and its absorbance was measured as follows: After capping, the beads (1 g) were suspended in a solution (10 mL) of 20% piperidine in DMF (dimethylformamide) and shaken for 30 min. The beads were then centrifuged, the supernatant was collected and its absorbance at 301 nm was measured. The number of reactive groups per bead (i.e., the bead loading) was calculated from

$$C = A/\epsilon l \quad (C = \text{conc. of Fmoc molecules})$$

$$\epsilon = 7800 \text{ for Fmoc at } 301 \text{ nm} \quad (1a)$$

Reactive amino groups/bead

$$= \text{Fmoc molecules/number of beads}$$

$$(\text{there are } 5 \times 10^{10} \text{ beads/g}) \quad (1b)$$

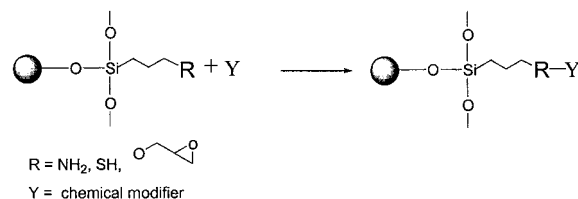
### Chemical Modifications of Silanized Beads

Figure 2 shows a general chemical modification scheme.

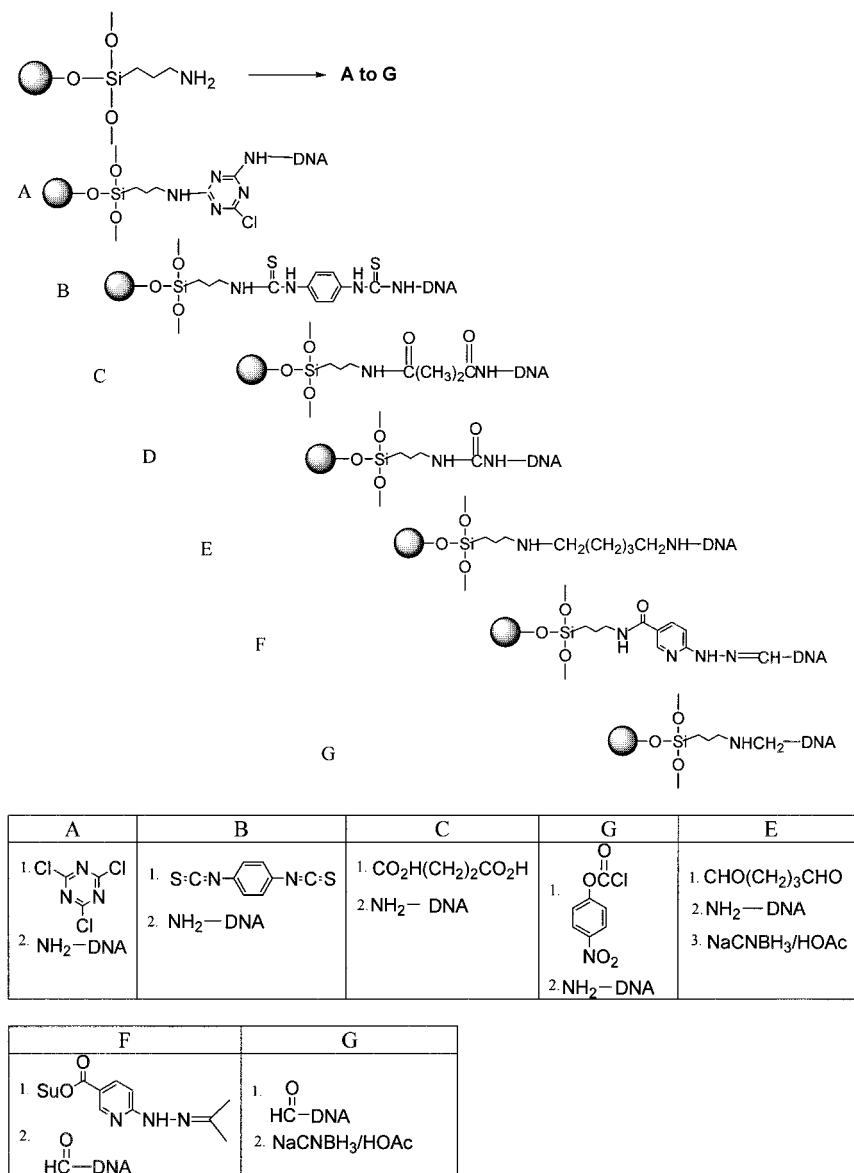
Detailed chemical modifications carried out on amino silanized beads are shown in Scheme 1.

Scheme 2 shows immobilization of thio-modified oligos on thiol-silanized beads.

Scheme 3 shows immobilization of amino-modified oligos on glycidoxypropyl-silanized beads.



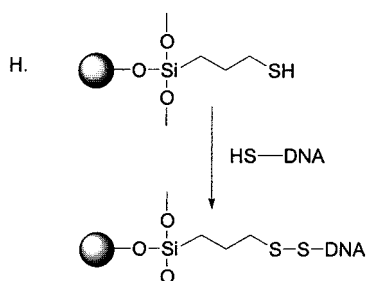
**FIGURE 2** General chemical modification of silanized beads.



SCHEME 1 Detailed chemical modifications of amino-silanzed beads.

**Cyanuric Chloride Activation.** Aminopropyl beads (100 mg) were suspended in acetonitrile (1 mL), followed by addition of 20  $\mu$ L (0.12 mmol) DIEA. After a brief

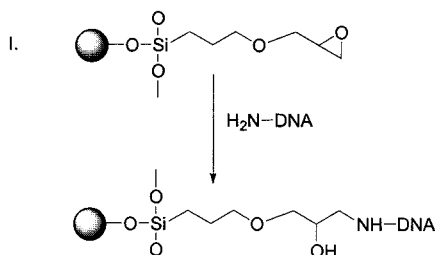
sonication, 10 mg (0.05 mmol) cyanuric chloride was added and the reaction mixture was shaken for 2 h at RT. The beads were washed 3 times with 1 mL acetonitrile and twice with 1 mL 0.05M sodium borate buffer (pH 8.5).



SCHEME 2 Immobilization of oligos on thiol beads.

**PDITC (1,4-Phenylene Diisothiocyanate) Reaction.** Aminopropyl beads (100 mg) were suspended in solution (10% pyridine and 0.2% PDITC in DMF, 1 mL) and shaken for 2 h at RT. The beads were washed 5 times with 1 mL DMF, 3 times with 1 mL ethanol, and 3 times with 1 mL methylene chloride.

**Dicarboxylic Acid Reaction.** Aminopropyl beads (100 mg) were dispersed in DMF (1 mL) followed by addition of 35  $\mu$ L DIEA (0.2 mmol). The suspension was sonicated, succinic acid (11.8 mg, 0.1 mmol) was added, followed by



**SCHEME 3** Immobilization of oligos on glycidoxypropyl beads.

addition of 52 mg (0.1 mmol) of PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate). The beads were shaken overnight at RT. They were then washed 3 times with 1 mL DMF, 3 times with 1 mL methylene chloride, and 3 times with 1 mL acetonitrile.

**Activation with 4-Nitrophenyl Chloroformate.** Aminopropyl beads (100 mg) were dispersed in methylene chloride (1 mL) followed by addition of 20  $\mu$ L DIEA (0.12 mmol), and 10 mg 4-nitrophenyl chloroformate (0.05 mmol). The beads were shaken for 2 h at RT and washed 5 times with 1 mL methylene chloride then 5 times with 1 mL acetonitrile.

**Dialdehyde Reaction.** Aminopropyl beads (100 mg) were dispersed in trimethyl orthoformate (TMOF) (1 mL), followed by addition of 5 mg glutaraldehyde (0.05 mmol). The beads were shaken for 30 min at RT and then washed 3 times with 1 mL DMF and 3 times with 1 mL ethanol.

**Reactions with Hydrazones.** Aminopropyl beads (100 mg) were dispersed in 1 mL buffer (100 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.3), followed by addition of 8.6 mg succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH) (0.03 mmol) in 80  $\mu$ L DMF. The suspension was shaken overnight and the beads were washed 2 times with 1 mL of the aforementioned buffer, and 2 times with 1 mL water. They were finally washed 2 times with 1 mL 0.1M sodium acetate buffer (pH 5.1) to deprotect the hydrazine.

## Oligo Immobilization

A general oligo immobilization scheme is shown in Figure 3.

**General Method for Oligo Immobilization.** HPLC-purified oligonucleotides (100 nmol in 0.05M sodium bo-

rate buffer, pH 8.5) were added to 2M sodium chloride solution (100  $\mu$ L). The oligo-NaCl solution was added to activated beads (25 mg beads in 100  $\mu$ L 0.05M sodium borate buffer, pH 8.5). The beads were shaken overnight, then washed 3 times with OmniPur water.

This immobilization method was directly applicable to activated beads from Scheme 1A, 1B, 1D, and Scheme 2. Immobilization of oligonucleotides onto other activated beads was done with slight modifications to the general method.

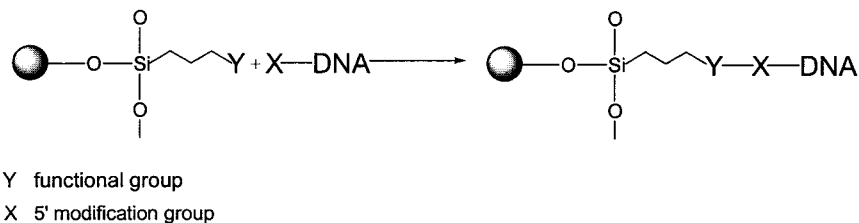
For beads coupled to dicarboxylic acid (Scheme 1C), 1M EDC solution [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride] was added during immobilization, to a final concentration of 100 mM EDC.

For beads coupled to aldehydes or dialdehydes (Scheme 1E, Scheme 1G), the immobilization itself was unchanged, but after it was complete, the unstable imine bond(s) were reduced to amine bond(s), as follows: Immediately following immobilization and washings, the beads (100 mg) were dispersed in 1 mL TMOF (trimethyl orthoformate), and a solution of sodium cyanoborohydride (3.4 mg, 0.054 mmol) in 100  $\mu$ L TMOF was added. The pH was then adjusted using acetic acid ( $\sim$ 90  $\mu$ L) to between 5 and 6. The suspension was shaken for 10 min, and the beads were washed 2 times with 1 mL DMF, 2 times with 1 mL methanol, 2 times with 1 mL 10% triethylamine in methylene chloride, and finally 2 times with 1 mL methanol.

For beads coupled to hydrazones (Scheme 1F), the immobilization procedure was carried out either in 0.05M sodium borate buffer, pH 8.5 (as previously described) or under acidic conditions, in sodium acetate buffer, pH 4.7.

For beads silanized with glycidoxypropyltrimethoxysilane (Scheme 3), 1M potassium hydroxide solution was added during immobilization, to a final concentration of 100 mM potassium hydroxide. Alternatively, Lewis acid catalyst such as zinc chloride (5 mg) was added instead of potassium hydroxide.

**Determination of Capture Probe Loading efficiency** 3'-Fmoc-modified 5'-amino-modified oligonucleotides were immobilized onto activated beads (25–100 mg), using cyanuric chloride chemistry as described above. After washing the beads with OmniPur water, 1 mL of 20% piperidine in DMF was added to the beads and the bead solution was shaken for 30 min. After centrifugation, the supernatant was collected and uv absorption was measured at 301 nm. The number of oligonucleotides on a bead was calculated using Eq. (1) as previously described. Figure 4 shows how the determination of capture probe loading efficiency was done.



**FIGURE 3** A general oligo immobilization scheme.

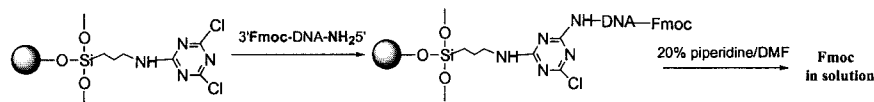


FIGURE 4 Determination of capture probe loading efficiency.

**Complementary Oligonucleotide (Target) Hybridization.** Hybridization of complementary oligo (target) to beads containing immobilized probes was carried out in solution and fluorescence-activated cell sorter (FACS) was used to measure hybridization intensity.

Hybridization was done in solution as follows: about 1 mg of probe-immobilized beads were suspended in 30  $\mu\text{L}$  hybridization buffer (0.1M potassium phosphate, 1M sodium chloride, 0.1% Tween-20, and 5% ethanol, pH 7.6), followed by addition of 15–20  $\mu\text{L}$  of  $\sim 1$  mM target (in 0.05M borate buffer, pH 8.5) and then shaken for 2 h. The beads were washed 2–3 times with hybridization buffer to remove excess target oligonucleotides. For the release of target from the beads, the beads were incubated with 50  $\mu\text{L}$  of 0.1M sodium hydroxide solution for 10 min.

### Detection of Hybridization

**Fluorescence Activated Cell Sorter.** Hybridization in solution was measured with a FACS instrument (Becton Dickinson Biosciences). We used constant laser power of 15 mW, and photomultiplier tube (PMT) gain of 659 for all measurements. Following hybridization in solution (described in the previous section), a small amount of beads was added to 1 mL OmniPur water (in FACS compatible test tube), and average fluorescence intensity was recorded for at least 10,000 beads.

**Determination of Hybridization Efficiency.** Ten milligrams of beads with immobilized oligonucleotides (capture probe) were hybridized with 50  $\mu\text{L}$  of 1 mM fluorescein-labeled complementary oligonucleotide (target) at room temperature for 1 h. After washing 10 times with OmniPur water, to remove excess target, the beads were incubated with 100  $\mu\text{L}$  of 0.1M NaOH for 10 min. After centrifugation, the solution was collected and diluted to a total volume of 1 mL with hybridization buffer. The fluorescence of the above solution was measured using a fluorometer (Perkin Elmer, Luminescence spectrometer LS50B). A standard fluorescence curve was established using different concentrations of a known amount of complementary oligonucleotide (target). The amount of dye-labeled oligonucleotide was then determined using that standard curve. This process is depicted in Figure 5.

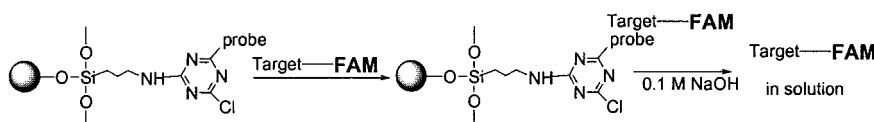


FIGURE 5 Determination of hybridization efficiency.

Hybridization efficiency was calculated from Equation 2:

hybridization efficiency

$$= \frac{\text{amount of dye labeled oligos(target)}}{\text{amount of probes on bead(probe)}}$$

## RESULTS AND DISCUSSION

### Covalent Immobilization of Capture Probe

The performance of the immobilization schemes was determined based on functional results, by measuring fluorescence intensity from target oligo (fluorescein modified) following hybridization to probe-functionalized beads. Maximal hybridization efficiencies or oligo loading were not investigated for each of the chemistries as separate entities. When choosing the optimal chemistry for bead arrays, we were more interested in hybridization intensities. All hybridizations were carried out in solution and detection was performed with FACS. Two different sequences (probes) were immobilized on the beads, and results of their hybridization are summarized in two separate tables. Results from immobilization of sequence 6 are summarized in Table I, and results from immobilization of sequence 5 are summarized in Table II.

All experiments (synthesis and immobilizations) were done in triplicate and the numbers (in tables) represent an average of these three readings.

Results in Table I and Table II show that four of the chemistry approaches give equally good hybridization intensities, within experimental errors: cyanuric chloride chemistry (Scheme 1A), diisothiocyanate chemistry (Scheme 1B), nitrophenyl chloroformate chemistry (Scheme 1D), and hydrazone chemistry (Scheme 1F).

Any of these four chemistry approaches can be used. All experiments and measurements described

**Table I Hybridization Results (on FACS) Using Probe Sequence 6 (Probe is Sequence 6: 5'-L-TTT GAA AAG GGA CGT GCG CTT CGA A-3')**

Chemistry	Scheme 1A	Scheme 1B	Scheme 1C	Scheme 1D	Scheme 1E	Scheme 2	Scheme 3
Counts	3500	3400	2700	3500	2300	1500	250
SD	200	210	247	215	198	130	17

below were performed using cyanuric chloride chemistry.

### Effect of Spacers on Hybridization Intensity

In theory, the more an immobilized oligonucleotide is spatially removed from the solid support, the closer it is to the solution state and the more likely it is to react freely with complementary oligonucleotides. To assess the influence of spacers of different length on hybridization properties of immobilized nucleotides, we used polyethylene glycol (PEG) linkers of varying length. The results of hybridizations to these spacer-containing beads are shown in Figure 6. It appears that the hybridization signal is not affected by the presence of spacer molecules.

### Bead Stability

Stability of the probe-immobilized beads, using cyanuric chloride activation, was tested over a period of five months. During that time period the beads were stored in ethanol at  $-20^{\circ}\text{C}$ . At each time point, aliquots of the beads were loaded on three fiber bundles and hybridized with the target for 10 min. After washing with hybridization buffer (to remove excess target) hybridization intensities were recorded using a CCD imaging system.<sup>14</sup> Results of this stability experiment are summarized in Figure 7. Hybridization intensities were very consistent and did not show any significant changes over the five-month period.

### Oligo Loading Optimization

To determine the number of amino sites available for probe immobilization, the beads were silanized by an

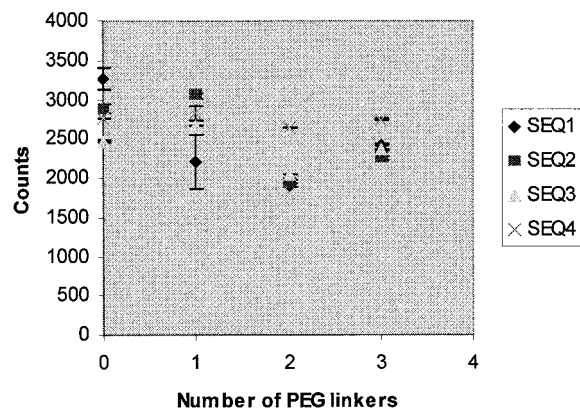
**Table II Hybridization Results (on FACS) Using Probe Sequence 5 (Probe is Sequence 5: 5'-L-TTT GAA AAG CCT ACA CGA CGG CGA A-3')**

Chemistry	Scheme 1A	Scheme 1F	Scheme 1G
Counts	3400	4000	1900
SD	241	300	176

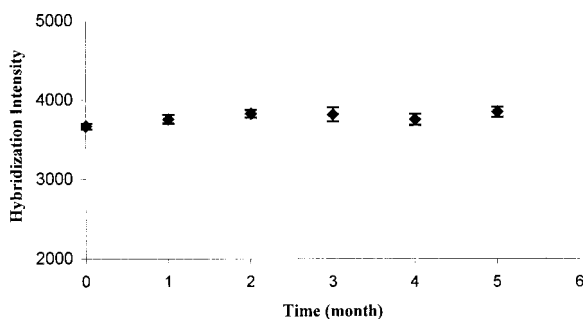
increasing concentration of silanization reagent: from  $5 \times 10^{-4}$  to 10%. The number of reactive amino sites measured was strongly dependent on the reactivity of 9-fluorenylmethyl chloroformate (see Materials and Methods for clarification). To obtain consistent results, a very dry reagent must be used. As the concentration of silanization reagent was increased from  $5 \times 10^{-4}$  to 1%, the amount of reactive amino sites grew, reaching a plateau at about 0.5% silanization reagent of  $6 \times 10^7$  amino sites per bead (Figure 8). Increasing concentration of silanization reagent past 1% did not increase further the number of amino sites per bead. Oligo loading was measured using covalent attachment of 3'-Fmoc-modified 5'-amino-modified oligonucleotides to the beads, as described in Materials and Methods. Calculations show that  $0.63 \times 10^6 \pm 0.086 \times 10^6$  probes per bead were immobilized. Even when  $>100$  times excess probes were used relative to the number of available reactive amino sites, the number of probes per bead did not increase. Thus, there are many amino sites that remain unreacted, suggesting that the limitations of oligonucleotide immobilization are a result of steric hindrance and not a lack of reactive amino sites.

### Packing Density

Packing density, at the point of optimal hybridization, was determined from the number of probes measured

**FIGURE 6** Effect of linkers on hybridization intensities of four random 25-mer oligonucleotides attached to the beads.

## Bead Stability



**FIGURE 7** Bead stability with time: (1) Beads with oligo attached were stored in EtOH at  $-20^{\circ}\text{C}$ . (2) At each time period, three fiber arrays were assembled and tested by hybridization. (3) Dyed beads were used to calibrate the intensity of the system. (4) During the five-month testing period, the coefficient variation (CV) of hybridization intensity was less than 10%.

at that point. As discussed in the previous section, there are  $0.6 \times 10^6$  probes per bead. The surface area of our beads, which are  $3 \mu\text{m}$  in diameter, is

$$SA = 4\pi r^2 = 4\pi(1.5 \times 10^{-6} \text{ m})^2 = 2.8 \times 10^{-11} \text{ m}^2$$

$$\begin{aligned} 0.6 \times 10^6 \text{ probes} / 2.8 \times 10^{-11} \text{ m}^2 \\ = 2.1 \times 10^{16} \text{ probes/m}^2 = 2.1 \times 10^4 \text{ probes}/\mu\text{m}^2 \end{aligned}$$

The number of probes in one  $\mu\text{m}$  of linear distance can be calculated by taking the square root of the probes/ $\mu\text{m}^2$ :

$$\begin{aligned} (2.1 \times 10^4 \text{ probes}/\mu\text{m}^2)^{1/2} \\ = 146 \text{ probes}/\mu\text{m} (\text{or } 0.0146 \text{ probes}/\text{\AA}) \end{aligned}$$

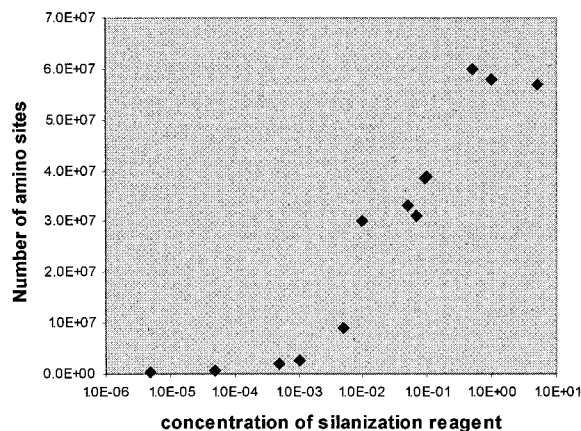
The center-to-center distance between two probes is therefore  $1/0.0146$  or  $69 \text{ \AA}$ . This packing density is at the limit of our packing capacity if we consider the fact that the length of our probes (25-mer oligonucleotides) plus the silanization reagent and cyanuric chloride molecules easily add up to  $90 \text{ \AA}$ . If the packing density is any higher, the probes will interfere with each other and hybridization efficiency will drastically decline. In our case we have a multilayer network of silanization reagent on the bead, which creates a rough surface. This allows the probes to be separated more than in the case of a smooth surface, thus resulting in a greater packing density than available on a smooth surface.

## Hybridization Efficiency

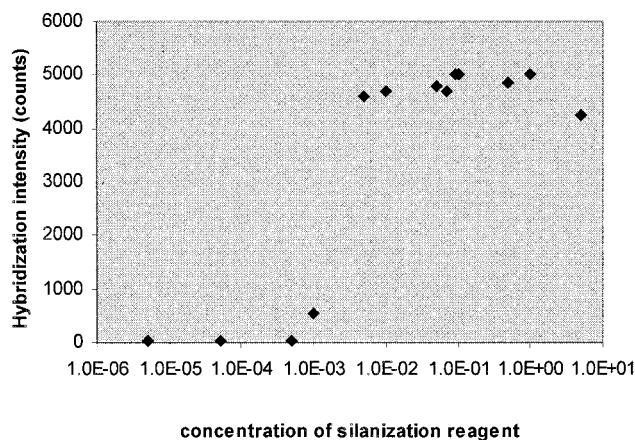
Packing density of the DNA capture probe is expected to be an important parameter affecting hybridization intensities. A low surface coverage would presumably yield a low hybridization signal. Conversely, high surface densities might result in steric interference between the covalently immobilized probes, impeding access to the target DNA strand. In principle, surface density is a measure of the number of probes immobilized per  $\mu\text{m}^2$ . However, packing density can also be correlated to the number of reactive amino sites on the bead (although not directly). The amount of reactive amino sites per bead can be controlled by varying the concentration of silanization reagent used. To evaluate surface densities and resulting hybridization efficiencies, we chose to alter the amount of bead silanization (i.e., amount of reactive amino sites) and follow the changes in hybridization intensities. Hybridization intensity is a product of two effects, packing density and hybridization efficiency, which are interdependent. Our goal was to find the point where the combination of these two gave the highest hybridization intensity.

It appears that optimal hybridization is observed when 0.005–1% silanization reagent was used, corresponding to  $4 \times 10^6$ – $8 \times 10^7$  reactive amino sites (Figure 9). We chose to use 0.5% silanization reagent for subsequent synthesis. The number of oligos per bead for 0.5% silanization was determined (see in Materials and Methods, the section on the determination of capture probe loading efficiency section) to be  $\sim 0.63 \times 10^6 \pm 0.086 \times 10^6$  oligos per bead.

Hybridization efficiency was then calculated at 0.5% silanization (with  $4 \times 10^6$ – $8 \times 10^7$  reactive amino sites available), as previously described in Materials and Methods, determination of hybridization



**FIGURE 8** Variation in the number of reactive amino sites as a function of concentration of silanization reagent.



**FIGURE 9** Hybridization intensity as a function of concentration of silanization reagent.

efficiency section. To describe this briefly: once maximum hybridization intensity was achieved, the hybridized beads were incubated with 0.1M sodium hydroxide solution for 10 min, and then the absorption of the supernatant was measured at 301 nm. Measurements indicate that there are  $0.25 \times 10^6$  target oligos hybridized at the point of maximal hybridization intensity. Accordingly, hybridization efficiency at that point is  $(0.25 \times 10^6 / 0.6 \times 10^6)$ , or about 45%.

## CONCLUSION

We evaluated several surface chemistries for use in attaching oligonucleotides covalently to silica beads. Four attachment methods produced derivatized beads that hybridized with high efficiency to complementary oligos. These were the cyanuric chloride, isothiocyanate, nitrophenyl chloroformate, and hydrazone methods. We chose to optimize the cyanuric chloride chemistry with regards to hybridization intensity. We investigated the stability of the derivatized beads over a five-month period and found no significant change in hybridization intensities. We also investigated the use of PEG spacer molecules to increase the distance of the oligos from the silanized bead surface, potentially reducing steric interference. We found that spacers had no effect on hybridization intensities. These results are in agreement with results obtained by Cantor et al.,<sup>12</sup> who did not observe an improvement in their hybridization efficiencies when they used a series of poly(dT) sequences as their spacers. They determined that 40% was the maximum hybridization that could be achieved. However, these results contrast with observations by Southern et al.,<sup>15</sup> who found in their system up to 150-fold increase in duplex yield when spacers were inserted between oligo-

nucleotides and a solid support. The discrepancy may be due to several factors, one of which may be the packing density. In our case there is a nominal distance of  $\sim 69$  Å between probes. However, we use a multilayer silanized surface, so that the actual distance between probes may be greater. Southern et al.<sup>15</sup> calculate about 40 Å between their probes on a flat surface. The higher packing density in this case may contribute to reduced hybridization yields, which can be improved by using spacers. The packing density in our experiments may already be optimized and therefore inserting spacers had no significant effect on hybridization yields.

It is likely that hybridization efficiency varies depending on the mode of attachment as well as on packing density. For example, when probes are synthesized directly on the surface (in situ synthesis) many truncated species are synthesized, which do not hybridize. For example, when McGall et al. synthesized 20-mer oligos in situ, they obtained only  $\sim 15\%$  yield of full-length oligos and observed hybridization efficiency of less than 10%.<sup>16</sup> We chose to immobilize full-length, presynthesized oligos and observe a hybridization efficiency of 45%. Our results are comparable to hybridization efficiencies observed by other groups that immobilized presynthesized oligos.<sup>12,17</sup>

The method used here is easily scalable to automated derivatization of beads in bulk. It provides a cost-effective means for manufacturing many thousands of different microarray elements.

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