12

Photoactivatable Silanes: Synthesis and Uses in Biopolymer Array Fabrication on Glass Substrates

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Abstract: We wish to report a fast and easy way to build hydrophobic layers and hydrophilic functional polymer layers thereafter onto glass surfaces. A benzophenone-based silane was synthesized and used to prepare stable, hydrophobic, photo-activatable coatings on glass supports. Hydrophilic polymers were then applied to the substrate, and photochemically cross-linked to the underlying silane. The resulting substrates had not only increased functional density due to plurality of functional groups on the polymer, but also enhanced stability against solvents and displacement reagents (such as water and phosphate salts). The substrates were suitable for fabricating oligonucleotide probe arrays either by in situ synthesis or immobilization methods.

1. Introduction

Non-porous flat solid substrates (e.g. glass substrates) have provided a format for manufacturing microarrays, which have revolutionized biological analysis in many ways such as miniaturization and parallel analysis. Biological materials, such as genes and antibodies, can be deposited in a precisely defined location. Small sample volume, high sample concentration and rapid hybridization or binding kinetics are possible to be achieved on such chip formats [1].

However, data quality and reproducibility will all rely on a stable and functional substrate. Many silylating agents produce coatings with undesirable properties including instability to hydrolysis and the inadequate ability to mask the silica surface which may contain residual acidic silanols. Methods have been developed for stabilizing surface bonded silicon compounds. For example, hydrophobic and sterically hindered silylating agents were described by Kirkland [2] and Schneider [3]. However, the use of these surface bonded silylating agents is disadvantageous, because they typically require very forcing conditions to achieve bonding to the glass, since their hindered nature makes them less reactive with the substrate.

Previously, high-capacity flat glass surfaces were prepared either by etching or coating with colloidal silica to increase the surface area and capacity[4-6].

In this case, the chemical nature of the glass surface and silane coating is the same as in the current flat glass supports. In another process, reactive polymer brushes were built on glass substrates using surface-initiated polymerization[7-9]. While this approach offers great potential, it is somewhat complicated and difficult to control. Recently, a method has been reported for grafting polystyrene films to glass surfaces using a photo-activatable silane[10]. Here we have adapted a similar approach for the attachment of polymers containing reactive functional groups which provide supports suitable for the synthesis and immobilization nucleic acids and other biomolecules. This paper describes the synthesis of the photoactivatable silanes, silanation on the glass surfaces, polymer coating and subsequent photoattachemt onto the surfaces. We also present photolithographic oligonucleotide synthesis on the surfaces and the hybridization of oligonucelotide target molecules. The polymer coated surfaces prepared by this method have the following advantages:

1. An initial hydrophobic silane coating offers protection from unwanted hydrolysis, and thus increased coating stability.
2. Polymer grafting provides multiple points of attachment to the substrate, which further increases the stability of the coating.
3. Plurality of functional groups on the polymer provides increased capacity for subsequent attachment of nucleic acid probes or other biomolecules, compared to conventional silanated flat substrates.
4. The polymer composition can be controlled in order to optimize properties for a given application (porosity, functional group content, intermolecular spacing, etc.).
5. Photolithographic oligonucleotide synthesis using MeNPOC chemistry [9] proceeds with substantially higher yield on these supports.

2. Materials and Experiments

GC-MS analyses were performed on Agilent 6890 GC System with 5973 MD detector. UV-Vis data were acquired on a Varian Cary 3E spectrophotometer. Proton NMR was recorded on a Varian Gemini-400 spectrometer. All reagents and anhydrous solvents were purchased from Sigma-Aldrich and used without further treatment, except for the following: MeNPOC polyethyleneglycol phosphoramidite, Pierce Biochemical (Milwaukee, WI); dimethyl-N,N-diisopropylphosphoramidite, 2-[2-(4,4′-dimethoxyltrityloxy) ethylsulfony]ethyl-(2-cyanoethyl)-(N,N-diisopropyl) phosphoramidite (5′-phosphate-ON reagent), ChemGenes (Waltham, MA); fluorescein phosphoramidite, 5′-DMT-dT 3′phosphoramidite, 5′-carboxyfluorescein phosphoramidite, 5′-MeNPOC 2′deoxynucleoside 3′ phosphoramidites, Amersham Pharmacia Biotech (Piscataway, NJ); C3 spacer phosphoramidite, DNA synthesis reagents and anhydrous acetonitrile,

Glen Research (Sterling, VA); Silica gel (60 Å pore size, 230-400 mesh) from E. Merck.

2.1 Synthesis of 4-(3′-Triethoxysily) Propylamidobenzophenone (APTSBP)

2.1.1 Preparation of Benzoylbenzoyl Chloride (BPCOCl)

Thionyl chloride (50 ml) was introduced into a three neck round bottom flask equipped with a condenser, drying tube and a gas bubbler under Ar. Benzoylbenzoic acid (BP-COOH) (13.8 g, 0.061 moles) was added to the flask. The suspension was stirred for half hour at room temperature and then slowly heat to reflux. Control the heating and watch for gas release. The BP-COOH was totally dissolved after 20 minutes of reflux. No more gas was generating after 40 minutes, indicating the reaction was complete. The mixture was stirred for one more hour after no more gas is generating. The excess thionyl chloride was removed by distillation. The minor left over was thoroughly removed under vacuum. Use two dry ice cold trap and NaOH solid trap to avoid damage to the pump. An oiless, Teflon paraphram pump would be recommended for this usage. A light yellowish solid (14.6 g) was obtained. Yield: 97%

2.1.2 Preparation of APTSBP

Aminopropyltriethoxysilane (13.2 g, 14 ml, 0.06 moles), triethylamine (6.06 g, 8.3 ml, 0.06 moles), and anhydrous tetrahydrofuran (20 ml) were introduced into a three neck round bottom flask under Ar. The mixture was cooled in an ice bath. BPCOCl (14.6 g, 0.061 moles) dissolved in 50 ml of anhydrous THF was added drop wise with good stirring. Fume of Et3N-HCl could be observed. White precipitate formed after 10 ml was added. The ice bath was removed after addition. The final mixture was further stirred for one hour, at the time the mixture was warmed up to room temperature. Prepare filtration equipment in glove box under dry nitrogen. The solid was filtered off. Wash with dry THF (10 ml). The filtrate was passed through silica gel (30 g, in a Buchner funnel) under vacuum. Wash the silica with THF (10 mL). The final filtrate was put on a rotavap to remove THF. A light yellowish solid was obtained and dried under vacuum. The process has a yield of 24 g at 92%.

GC-MS: >95% purity, 429 (M+).

[1] HNMR: (CDCl3, 400MHz), 0.75 (t, 2H,), 1.2 (t, 9H), 1.8 (quintet, 2H), 3.5 (quartet,2H), 3.8 (quartet, 6H), 6.8 (br, 1H), 7.5 (t,1H), 7.6 (d, 2H), 7.7 (d,2H),7.8 (d, 2H),7.9(d,2H).

2.2 Preparation of Hydrophobic and Photoactivable Layers on Glass Surfaces

Glass slides were cleaned by soaking successfully in Nanostrip (Cyantek, Fremont, CA) for 15 min, 10% aqueous NaOH/70 0C for 3 min, and then 1% aqueous 1% HCl fro 1 min, rinsing thoroughly with deionized water between each step, and then spin drying for 5 min. under a stream of nitrogen at 35 0C. The slides were then silanated for 1 hr in a gently agitating 1% solution of APTSBP in toluene, rinsed thoroughlywith toluene, then isopropanol, and finally dried under a stream of nitrogen.

2.3 Preparation of Functional Polymer Layers

A thin layer of polymer coat on glass surface was performed by spin-casting solutions of polymers at a spin speed of 2000 rpm for 20 second. Typical polymer solution were made in deionized water at 1% concentration by weight. The spin coated slides were dried in a 50 0C degree oven for 30 min and cooled down to room temperature before illumination. The illumination was carried out in a BioLink UV box at 254 nm, for 15 min. with a total energy of 2.2 joules. The slides were sonicated in water bath for 2 minute, then soaked in water at room temperature overnight. We rinsed slides with water and iso-propanol, then dried with a stream of nitrogen. The thickness of the resulting polymer layers were determined by ellipsometry.

2.4 Substrate Stability Test by Surface Fluorescence

A substrate slide was mounted onto a flow cell connected with an Affymetrix Arrray Synthesizer, following a standard synthesis cycle as described by McGall et al [11]. First the MeNPOC polyethyleneglycol phosphoramidite was coupled on the substrate and capped with dimethyl-N,N-diisopropylphosphoramidite. The substrate was washed with acetonitrile and dried with Ar. A strip patterned mask was used to mask the substrate and a 365 nm light source was applied (total 6 Joules) for the photolysis. A mixture of fluorescein phosphoramidite (0.5 mM) and DMT-dT phosphoramidite (49.5 mM) was introduced to react with the hydroxyls released from photolysis. The fluorescein was deprotected by ethylenediamine:ethanol (1:1) at room temperature for one hour. The substrate surface fluorescence intensity was acquired using a scanning confocal fluorescence microscope with photon-counting electronics. The intensity values are proportional to the amount of surface-bound fluorescein. The functional group density could be determined by direct comparison of the observed surface fluorescence intensity. Surface fluorescence within the nonilluminated regions of the substrate was taken as nonspecific background. To test the stability of the substrate, the scanned slides were soaked in a 0.5 M sodium chloride

0.1 M sodium phosphate, 10 mM EDTA, 0.01% Triton, pH 7.8 buffer (SSPE) for 17 hours at 45 ˚C. The slides were rinsed with water and re-scanned.

2.5 Hydroxyl Site Density Measurement

Fluorescein tagged molecules were synthesized on the glass surface on an Affymetrix Array Synthesizer using phosphoramidite chemistry. A cleavable linker (5′-phosphate-ON reagent) was synthesized on the surface, followed by a spacer (C3 spacer phosphoramidite) and 5′carboxyfluorescein phosphoramidite). The surface was then diced into about 1 cm2 pieces, weighed, placed in a glass vial and cleaved into 1.0 ml of ethylenediamine:water (1:1) containing internal standard at 50 ˚C for 4 hours. The released 3′pC3-fluorescein was analyzed by a Beckman System Gold HPLC with an ion-exchange column and fluorescence detector. The internal standard 3′pC3C3-fluorescein was made separately on an ABI synthesizer and quantified by UV-Vis.

2.6 Photolithographic Synthesis of Oligonucleotides on Prepared Substrates

Oligonucleotides were synthesized on an Affymetrix Array Synthesizer using standard DNA synthesis cycles as described by McGall et al [11]. Photodeprotection was performed with an open square mask using 6 joules of 365 nm irradiation. All phosphoramidites were used at a concentration of 50 mM in anhydrous acetonitrile. The sequence of coupling is the following: a spacer, the MeNPOC polyethyleneglycol phosphoramidite; unreacted hydroxyls capping by dimethyl-N,N-diisopropylphosphoramidite; a cleavable linker, 2-[2-(4,4′-dimethoxyltrityloxy)ethylsulfony]ethyl-(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite; a fluorescent label, 5-carboxyfluorescein phosphoramidite; and then a sequence of 5′-MeNPOC 2′-deoxyribonucleoside phosphoramidites. After synthesis, the entire synthesis area (about 1 cm^2) was diced, placed in a glass vial, and cleaved into 1.0 ml of ethylenediamine:water(1:1) containing internal standard at 50 ˚C for 4 hours. The cleaved products were analyzed by a Beckman System Gold HPLC with an ion-exchange column and fluorescence detector. Elution had a linear gradient of 0.4 M $NaClO_4$ in 20 mM Tris pH 8.0 buffer at a flow rate of 1.0 ml/min.

To determine the efficiency of oligonucleotide synthesis, a relative synthesis yield was calculated by dividing the integrated area of the full length oligo peak by the total area of all products cleaved from the surface.

2.7 Target Hybridization to Probes

Oligonicleotide probes (20mer) were synthesized in a checkerboard pattern on Affymetrix Array Synthesizer using photolithography and phosphoramidite

chemistry as described by McGall et al [11]. The removal of protecting groups was performed by soaking the slides in ethylenediamine:ethanol (1:1 by vol) solution for alt least 4 hrs at room temperature. A typical probe sequence (from the surface) was 3′-GACTTGCCATCGTAGAACTG-5′. The 5′-fluorescein-labeled oligo-nucleotide target had a concentration of 10 nM in a hybridization buffer containing 0.1M MES (2-[N-morpholino]ethanesulfonic acid, 0.89M NaCl, and 30 mM NaOH, pH 6.8). This target concentration is to saturate surface probes. The hybridization was carried out by soaking the slides in the target solution at 45 ˚C for 17 hrs with gentle agitation. The slides were scanned on a confocal fluorescence microscope after extensive washes of the slides with fresh hybridization buffer.

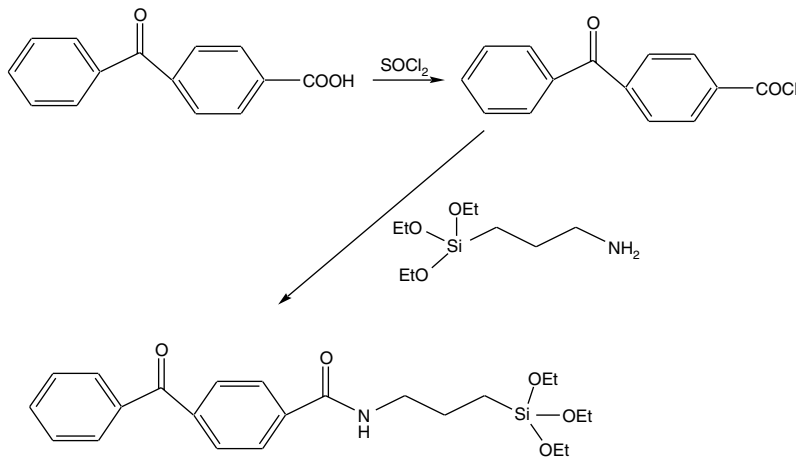
To evaluate hybridization discrimination, probe sequences on the surface were designed to include perfect matches, a single-base mismatches at base 10 (A substituted for T) and two-base mismatches at both bases 10 and 12 (A substituted or G). The discrimination ratio was defined in the following equation in which the Fpm is the fluorescence signal intensity for perfect match regions and Fmm is the signal intensity for mismatch regions after background correction).

$$Rd = (Fpm - Fmm) / (Fpm + Fmm)$$

3. Results and Discussion

3.1 Photoactivatable Silane

Shown in Scheme 12.1, the benzophenone moiety was coupled to a typical silane compound with high purity and high yields. A quick filtration through



SCHEME 12.1. Synthesis of (4-(3′-triethoxysily) propylamidobenzophenone) (APTSBP).

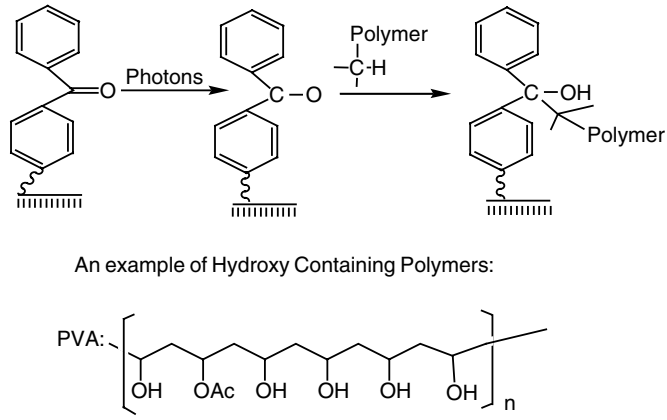


FIGURE 12.1. Photocrosslinking of polymers onto the benzophenone layers.

a short dry silica gel column provided a sufficient purification on the product. The purity and structure identity were confirmed by GC-MS and proton NMR. This synthetic strategy could be readily extended to make a variety of photoactivatable silanes, including benzophenone and azido compounds.

The photochemistry of benzophenone is depicted in Figure 12.1. The benzophenone structure was chosen for several advantages. It is not only chemically inert and stable as well under ambient light. It has well-characterized photochemistry and the light generated intermediates non-selectively insert to C-H bonds in a wide range of different chemical environments. Water molecules do not affect its photoactivity due to the reversibility of the mechanism in contrast to the water quenching problem in nitrene (azido) system [12].

The process is depicted in Figure 12.2. The benzophenone layer was first immobilized on glass surfaces using standard silanation. The molar concentration of the silane is aout 20 mM in anhydrous toluene. A base catalyst triethylamine is also added to a final concentration of 20 mM. This anhydrous procedure gives monolayers of the benzophenone silane on the glass surface [13].

Prior to photocrosslinking, the polymer was spin-coated on the glass surface. The polymer concentration of 1% by weight in water gave a thin layer coating on the top of benzophenone silanes. In the spin-coating process, it was found the polymers need to have surfactant effect, so that the polymer solutions had low surface tension to be able to spread on the hydrophobic benzophenone silane surfaces. Polyvinyl alcohols and polyacrylamide derivatives all were found to have those required properties. The polymer and benzophenone silane layers were then illuminated with UV light at 254 nm. Benzophenone has a maximum absorption around 260 nm. The intermediate is actually a biradical triplet. It either inserts to C-H bond in its vicinity or returns to the ground state which can be reactivated by light. This nature

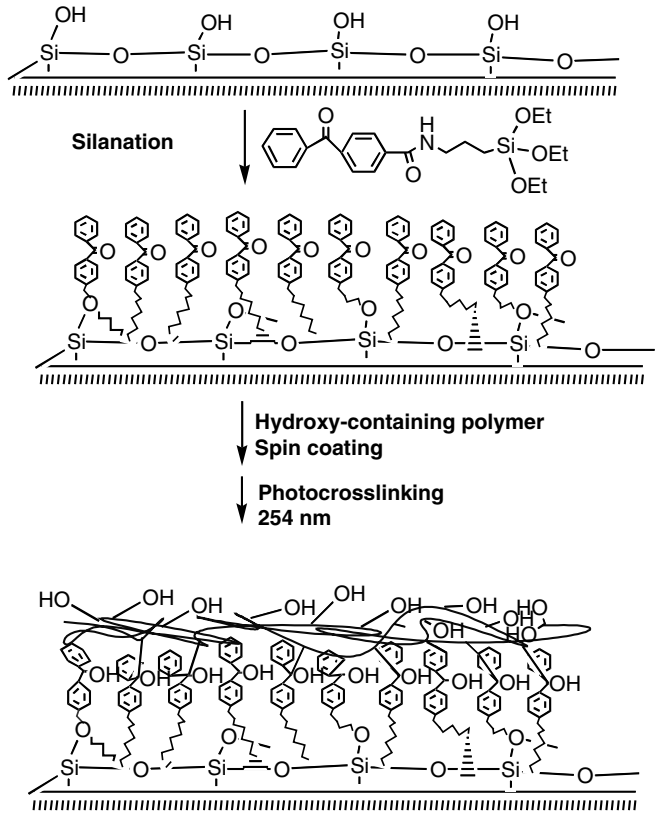


FIGURE 12.2. Process of polymeric surface on glass substrate

allows benzophenone to have higher photoattachment efficiency. The thickness of the hydrophobic layer and the polymer layer was measured at 13 Å and 34 Å respectively, each corresponding to a monolayer of molecules (Figure 12.3).

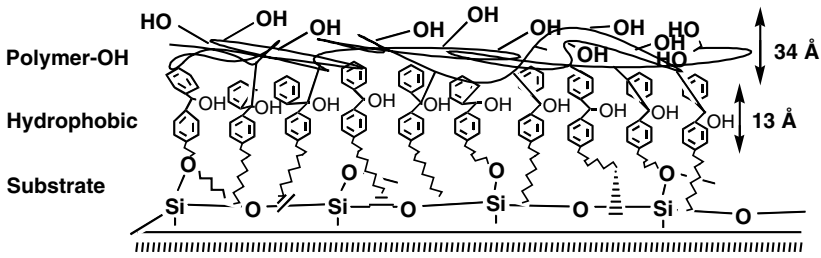


FIGURE 12.3. Thickness of the multilayered polymer surface.

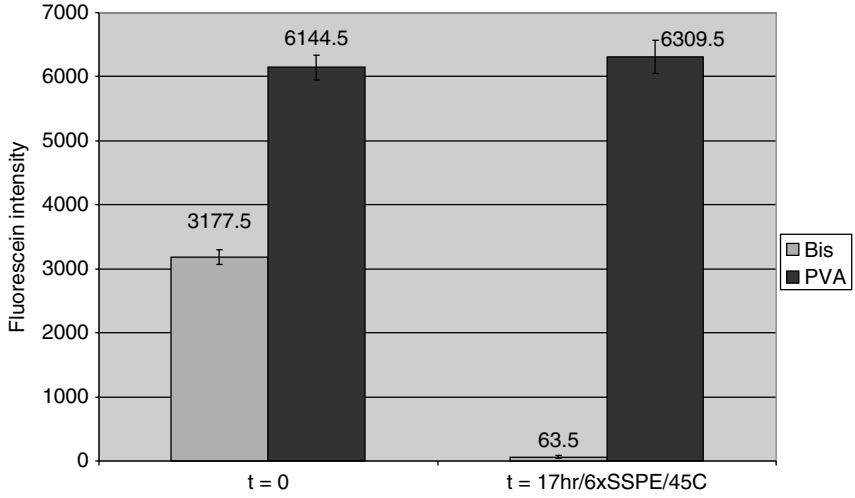


FIGURE 12.4. Substrate stability comparison by surface fluorescence analysis. Bis suface is a standard silence surface. PVA is the polymeric surface.

3.2 Stability of the Polymeric Surfaces

Surface fluorescence analysis was employed to test the substrate stability. The stability comparison is shown in Figure 12.4. The polymeric surfaces are stable under harsh hydrolysis conditions. The instability and loss of functional groups on the substrate are due to hydrolysis of siloxy bonds formed on the surfaces. Again the hydrolysis released silanol can go back to the surface to form siloxy bond. There exists an equilibrium of siloxy bond hydrolysis and reformation on the surface. The polymeric substrate prepared in this paper has the following characteristics. Multiple attachment points better prevented the loss of functional groups. On the other hand, the hydrophobic nature of silane layers efficiently protected the surface from water attack.

3.3 Hydroxyl Site Density and Oligonucleotide Synthesis Efficiency

Figure 12.5 shows the procedures for quantification of surface hydroxyl density and analysis of oligo synthesis efficiency. The cleaved products are quantified by HPLC. Comparisons of site density and synthesis efficiency between standard silane surface and the polymeric surface are summarized in Table 12.1.

Chromatograms of T6mer and mixed base oligo16mer synthesis were shown in Figures 12.6 and 12.7. The 16mer oligo sequence is GAATGACATTTACAGC. The hydroxyl site density on PVA surface is 30% higher than that on standard silane surface. T6mer synthesis efficiency improved by 55% and mixed base oligo16mer synthesis efficiency improved 62%. These results

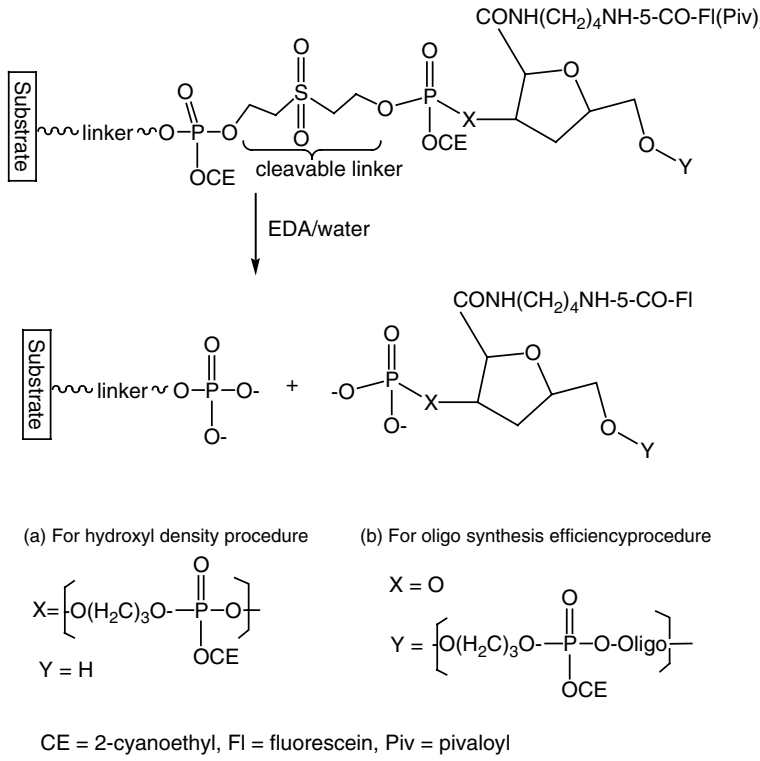


FIGURE 12.5. Procedures for surface hydroxyl density quantification and oligo synthesis efficiency analysis.

TABLE 12.1. Comparison of Site Density and Efficiency

Glass substrate	Hydroxyl density pmoles/cm^2	Relative T6 synthesis yield (%)	Relative oligo16mer synthesis yield (%)
Standard	141.2	31.3	6.3
PVA	182.8	48.5	10.2

clearly indicate the hydroxyl groups on the polymer are more accessible for extended oligonucleotide synthesis off the glass surface. It is expected that longer oligonucleotide synthesis will have bigger improvement on PVA surface versus standard silane surface.

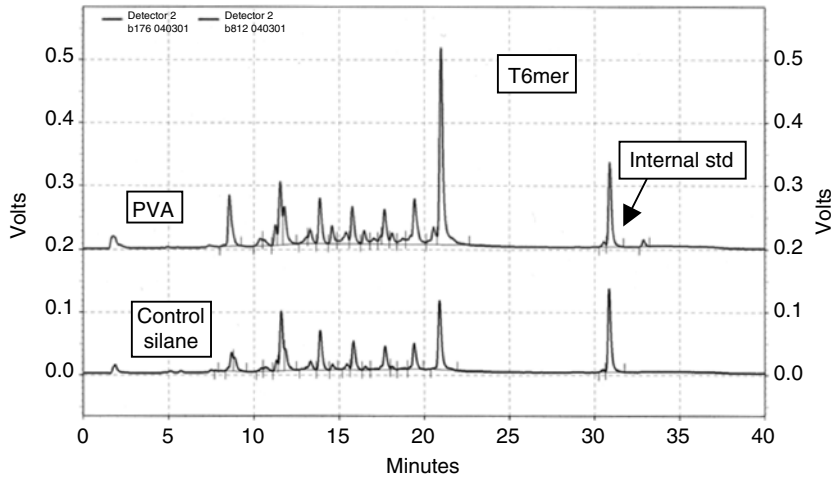


FIGURE 12.6. Synthesis of Oligonucleotides (T6mer) Comparison between standard silane and polymer surface.

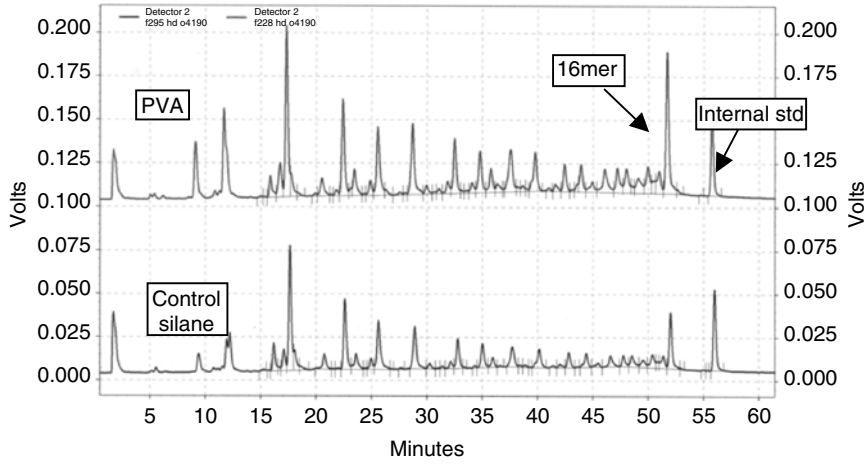


FIGURE 12.7. Synthesis of Oligonucleotides (16mer GAATGACATTTACAGC) Comparison between standard silane and polymer surface.

3.4 Hybridization and Discrimination

PVA surface gave hybridization signal more than four times higher than the standard silane surface after background correction. Both PVA and standard silane surfaces have similar discrimination ratio. The hybridization signal and discrimination ratio were shown respectively in Figure 12.8 and 12.9. Fpm, Fmm10, Fmm12, Fmm1012 are hybridization signal at perfect match, single

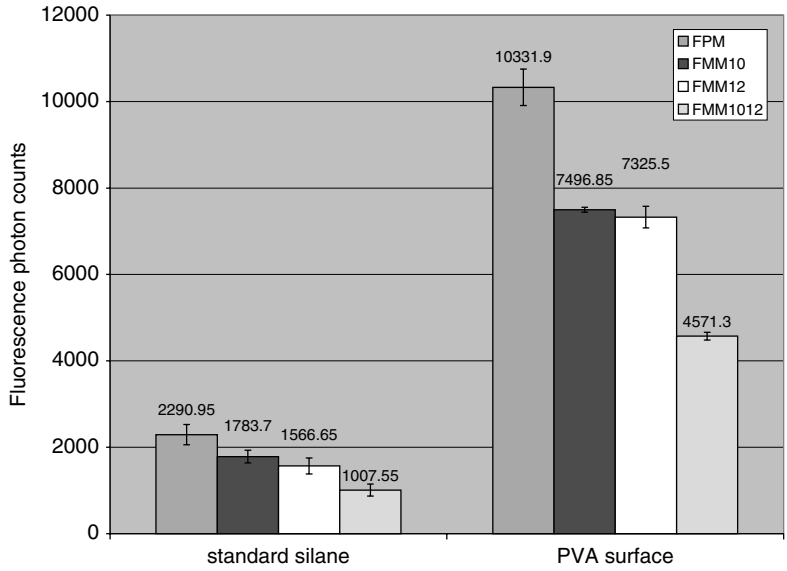


FIGURE 12.8. (also see the text).Hybridization (10 nM target in MES buffer at 45 0C for 17hrs).
Probe: 5′-GTCAAGATGCTACCGTTCAG-3′.
Target: 3′-CAGTTCTACGATGGCAAGTC-fluorescein.

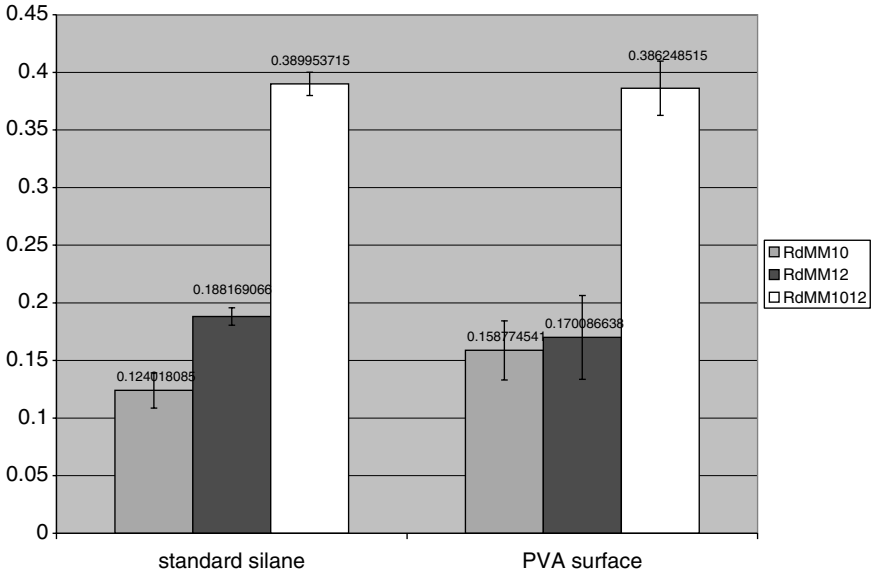


FIGURE 12.9. Discrimination ratio.

mismatch at base 10, single mismatch at base 12 and two mismatches at base 10 and 12. Rdmm10, Rdmm12 and Rdmm1012 are discrimination ratio for single mismatch at base 10, single mismatch at base 12 and two mismatches at base 10 and 12.

4. Conclusions

A photoactivatable silane was synthesized, characterized and used to photocross link functional polymers onto glass substrate. The layer of silane was hydrophobic thus to protect the surface from water and salt attack. Multiple crosslinking points of polymer macromolecule also enhance molecular attachment on the surface. They together provided a superior surface stability. The functional groups like hydroxyls on the surface bound polymer were more accessible to in situ synthesis of oligonucleotides with a 50-60% improvement on relative synthesis yield. The 20mer oligonucleotide probes synthesized on the polymeric surface were able to hybridize the complimentary target oligoncleotide with a similar specificity to what obtained on standard silane treated surface.

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