Aminopropyl Embedded Silica Films as Potent Substrates in DNA Microarray Applications

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ABSTRACT

Sol-gel derived silane hybrid films composing of (3-aminopropyl)trimethoxysilane–tetramethoxysilane (APTMS-TMOS) have been shown to have properties that make the films suitable for DNA microarray applications. The ability of the films to bind DNA was studied using aminated 25-mer oligonucleotide DNA and 1,4-phenylenediisothiocyanate linker. The binding of DNA onto the films was shown to depend on films’ composition, i.e. the binding rate decreased with the decrease of APTMS content in the film. The arrayed primer extension (APEX) analysis showed reversed tendency – fluorescence signals of bound probes increased with the decrease of APTMS content in the film. The background noise was low and the fluorescent probes were clearly outlined. The density of functional amino groups on the films was estimated by fluorescence spectroscopy.

INTRODUCTION

DNA microarrays are devices displaying specific oligonucleotides or longer DNA fragments attached in two-dimensional order onto activated solid surface [1]. DNA microarrays permit the analysis of gene expression and DNA sequence variation in a massively parallel format. The physical and chemical nature of the substrate on which the reactions are performed is one of the key factors influencing the quality and reproducibility of the results. Among many different types of substrates for DNA microarray analysis, the most common chemical treatments provide chemically reactive amine or aldehyde groups prepared by silanization. Despite being widely used, silane-treated slides lack the desired reproducibility – a fact that has driven a constant search for chemically alternative techniques rather than improvements of silanization protocols.

In the present study we focus on fabrication of APTMS-TMOS hybrid films in search of new and improved substrates for DNA microarray analyses. We utilised the idea of gelling pre-polymerised APTMS sol on glass substrate, where homogeneous and uniform siloxane surfaces were formed [2]. Now, for improvements in cross-linking between individual siloxane particles as well as optimising the surface density of amino groups, APTMS was co-polymerised with TMOS. The potential of the films to act as substrates in DNA microarray analyses is discussed in comparison with their commercial analogues (SAL-1 slides, Asper Biotech [3]) in terms of DNA binding and APEX assays.
EXPERIMENTAL DETAILS

Preparation of APTMS-TMOS films

APTMS ((3-aminopropyl)trimethoxysilane, (CH₃O)₃SiCH₂CH₂CH₂NH₂) and TMOS (tetramethoxysilane, Si(OCH₃)₄) (both Sigma-Aldrich) were mixed at molar ratios 1:1, 1:3, 1:5, and 1:7, respectively. Then, at room temperature and constant stirring, a mixture of water/methanol (Naxo, analytical grade) was added dropwise to the mixture of silanes. The final molar ratio of (APTMS+TMOS)/H₂O/MeOH was kept as 1:2:2. The mixtures were stirred until they turned to highly viscous spinnable matter (ca 30 min). Then, the polymerisation reaction was suppressed by introducing cold dry methanol to the mixture of silanes, thus making up the final molar ratio of (APTMS+TMOS)/MeOH 1:7. The final product was kept sealed at 4°C as stock solutions.

Before silanization, the glass slides (75 mm x 25 mm x 1 mm, Waldemar Knittel Glasbearbeitungs GmbH & Co KG), were washed thoroughly prior to use with acetone, 3 M NaOH solution in 1:1 v/v mixture of water/95% ethanol and finally with distilled water. Then the stock solution of silanization reagent was diluted 10 times with dry methanol and 100 µl of it was dropped to the centre of the glass slides spinning horizontally at 2300 rpm. Thereafter the slides were kept in open air (relative humidity 30%) for 48 h, and subsequently the temperature was raised to 120°C (0.3°C/min) and kept there for 12 h.

The binding of DNA and APEX assays

The silanized slides were gently shaken in 0.02 % w/w 1,4-phenylenediisothiocyanate (PDC) (Sigma-Aldrich) solution in 10% w/w pyridine/dimethylformamide (Fluka, analytical grade) for 2 h, which activated the slides for immobilization of amino-modified DNA. Then the slides were thoroughly washed with acetone, methanol and ethanol (Naxo).

For DNA binding experiments 1 part of 3’ Cy3-labelled 5’-aminomodified 25-mer oligonucleotide DNA (MWG-Biotech) was diluted 100 times with corresponding unlabelled 25-mer oligonucleotide DNA (MWG-Biotech). The mixture was spotted to glass slides with Virtek Chip Writer Pro spotter (now Bio-Rad) in 100, 80, 50, 30, 10, 3, 1, 0.4 and 0.1 micromolar series, respectively. For dilutions Genorama Spotting Solution Type I (Asper Biotech) was used. The spotted slides were incubated in humid air at 37°C for 2 h and subsequently treated with ammonia vapour for 1 h for blocking, washed thoroughly with hot distilled water and dried by centrifugation at 280 g for 3 min. For comparison, SAL-slides (prepared by incubation of cleaned glass slides in 2% w/w APTMS solution in 95% w/w acetone/water for 2 min and activated with PDC) were processed in the similar manner. The fluorescence of DNA spots was measured using ScanArray 5000 (Perkin-Elmer) microarray scanner. The spots were analyzed with Genorama Genotyping Software 4.1 (Asper Biotech).

APEX reactions were studied in two ways using both synthetic and native DNA templates. For APEX reactions with synthetic DNA templates 14 different 5’amino-modified 25 mer oligonucleotide probes were spotted onto both standard SAL- and APTMS-TMOS slides in 50 µM concentration dissolved in Genorama Spotting Solution Type I (Asper Biotech) with Virtek Chip Writer Pro spotter (Bio-Rad). For these 14 probes 56 synthetic DNA templates (four per probe) were designed and ordered (MWG Biotech). To evaluate the APTMS-TMOS slides for APEX with native DNA oligonucleotides probes specific to 226 human mutations were spotted.
in a similar way. The target DNA fragments were PCR-amplified from human DNA and prepared for APEX reactions as described in [4]. One-sixth of every amplification product or 5-10 µg of synthetic template DNA was utilized in the primer extension reaction on the microarray. Each APEX reaction consisted of a fragmented and denatured PCR product, 4 U of ThermoSequenase DNA Polymerase (GE Healthcare Amersham), 1 x reaction buffer and 1.4 µM final concentration of each fluorescently labeled ddNTP: Texas Red-ddATP, fluorescein-ddUTP (GE Healthcare Amersham), Cy3-ddCTP, Cy5-ddGTP (Perkin Elmer). The reaction mixture was applied to microarray slide for 15 min at 58°C. The reaction was stopped by washing the slides at 95°C in Milli-Q water. The slides were imaged with the Genorama QuattroImager detector (Asper Biotech) and the sequence variants identified by Genorama Genotyping Software [5].

**Determination of the content of amino groups on the surface of the films**

For estimation of the density of amino groups on aminated slides 11 SA slides (treated as described above, but not activated with PDC) (Asper Biotech) were incubated for 5.5 h in 42.7 g of 1.89 µM fluorenone solution in dichloromethane (both Sigma-Aldrich). The fluorescence spectra of the fluorenone solution were collected before and after the incubation. A Nd:YAG pulsed laser (Laser Compact) operating at 355 nm was used for excitation of fluorescence. The spectra were recorded by a spectrograph equipped with an ICCD detector (Andor). The decrease in the fluorenone concentration (fluorenone couples covalently to NH₂ groups) was assigned to correspond to amino groups on the surface, which considering the overall surface area of 11 slides let us calculate the density of amino groups. In order to minimize the non-specific binding of fluorenone the solution with glass slides was sonicated for 5 min before acquiring the spectrum.

**RESULTS AND DISCUSSION**

Schematically, the formation of APTMS-TMOS hybrid films can be drawn as:

\[ \text{SiOH} + \text{SiOH} \rightarrow \text{Si-O-Si} \]

The density of amino groups on the surface could be varied by changing the initial ratio of APTMS/TMOS. This enabled us to change the maximum amount of immobilized DNA as well as the outcome of APEX analysis as correlation between the composition of the films and the binding of DNA was confirmed.

**The binding of DNA**

The binding of DNA to APTMS-TMOS films is presented in figure 1. It can be seen that the maximum binding decreased with the decrease of APTMS content in the films, but was not proportional to it.
Figure 1. The maximum binding of DNA to APTMS-TMOS films. The binding to SAL-slide was 13500±3200 rfu.

The result suggest that the degree of surface heterogeneity was comparable in the case of hybrid films, e.g. the increase in APTMS content in the film yields higher affinity to DNA, but is limited to the steric saturation of the surface. Compared to SAL-slide, the binding to APTMS-TMOS films was significantly lower (caption to figure 1). Obviously, the surface heterogeneity of SAL-slides was different from that one of the hybrid films, suggesting higher specific surface area and more dense coverage of active amino groups. Since SAL-slides are silanized from dilute basic solutions, the formation of granular siloxane particles is favored [6]. These particles adsorb to glass surface and form a porous film with granular texture [2]. Consequently, in terms of DNA binding capacity SAL-slides are 5-10 times more efficient.

APEX analysis

The results of APEX reactions with synthetic DNA templates are shown in figure 2. Primer extension was working with all four fluorescently-labeled terminator nucleotides. The fluorescence intensities were comparable in case of ddATP, ddCTP and ddGTP terminators labeled with different fluorophores for APTMS-TMOS films at concentrations of 1:5 and 1:7, and SAL control slides. Variations in fluorescein-labeled ddUTP signals between different channels could be attributed to optical instability of fluorescein dye. In the case of APTMS-TMOS 1:3 film the fluorescence intensities were weaker compared to 1:5 and 1:7 films. This could be explained by steric hindrance to primer extension process, since APTMS-TMOS films have denser coverage of primer DNA (see fig. 1). Obviously, the optimal primer surface density did not correspond to the ultimate one, but remained lower. High signal fluorescence intensities in the case of SAL-slide could be assigned to higher topographical surface density compared to hybrid films (see previous section), a fact that makes these slides effectively to bind primer DNA, but which largely remains ineffective in terms of primer extension (see fig. 1,2).

As a result of APEX analysis with native DNA templates it was shown that all 226 mutation-specific oligonucleotides probes were working on both standard SAL-slides and APTMS-TMOS slides (data not shown). However, on APTMS-TMOS slides fluorescence signal intensities were 3 to 4 times weaker compared to standard SAL-slides. Also, the fluorescence signals on
The density of amino groups on aminated slides

The spectra showing the decrease of fluorenone concentration in the system (see Experimental Details) are shown in figure 3. This corresponded to the adsorption of about $5 \times 10^5$ fluorenone molecules per $\mu m^2$ on SA-slides. Considering the DNA binding data (figure 1) it allowed us to assume that the density of active...
amino groups ranged between \(1 \times 10^5\) to \(0.5 \times 10^5\) \(\text{NH}_2/\mu\text{m}^2\) for APTMS-TMOS 1:1 film to APTMS-TMOS 1:7 film, respectively.

The longwave absorption and fluorescence excitation of fluorenone in various solvents (e.g. hexane, THF, EtOH and others) has been thoroughly discussed in [7]. Fluorenone couples covalently to \(\text{NH}_2\) groups, thus, the idea of measuring the decrease of fluorenone concentration while incubating amino slides in fluorenone solution provides a simple and rapid estimation of the amount of amino groups in the system. The results indicate that the binding capacity of SA-slides is significantly higher than that one of the hybrid films. For explanation one has to bear in mind that SA-slides are silanized from dilute silane solutions, where in basic medium three-dimensional nanosized siloxane particles evolve [6] and form dense packing on the glass surface. On contrary, sol-gel films are congealed gels with uniform and smooth surface topography [2] and thus, possess little specific surface area. Considering the peculiarity of biochemical reactions taking place on the film’s surface this fact may not be a drawback. Because of the steric hindrance the maximum surface density of reactive groups do not necessarily correspond to optimal reaction conditions [8] as it was evidenced in the case of the APEX assay (see figure 2).

CONCLUSIONS

This study shows that APTMS-TMOS hybrid films can be used as substrates in DNA microarray analyses. The maximum binding of DNA to hybrid films decreased with the decrease of APTMS content in the film. The binding capacity of hybrid films was 5-10 times lower than that one of commercial SAL-slides. The surface density of active amino groups was in range of \(0.5 \times 10^5 – 1 \times 10^5\) \(\text{NH}_2/\mu\text{m}^2\). APEX analysis performed on APTMS-TMOS films indicated that the films were suitable for practical applications including mutation screening. The flexibility of the procedure for silanization and low fluorescence background of the films are of great importance in terms of increased reliability of DNA microarray analyses.

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