

Sol–gel films for DNA microarray applications

K. Saal^{a,b,*}, T. Tätte^{a,b}, I. Tulp^b, I. Kink^a, A. Kurg^c, U. Mäeorg^b, A. Rinke^b, A. Lõhmus^a

^a Institute of Physics, University of Tartu, 142 Riia St., 51014 Tartu, Estonia

^b Institute of Organic and Bioorganic Chemistry, University of Tartu, 2 Jakobi St., 51014 Tartu, Estonia

^c Institute of Molecular and Cell Biology, Estonian Biocentre, 23 Riia St., 51010 Tartu, Estonia

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Abstract

Sol–gel derived (3-aminopropyl)trimethoxysilane–tetramethoxysilane ((CH₃O)₃SiCH₂CH₂CH₂NH₂–Si(OCH₃)₄) hybrid films are shown to have properties that make the films suitable for DNA microarray applications. The essential characteristics of the films are discussed on the basis of binding of aminated 25-mer oligonucleotide DNA to the films via 1,4-phenylenediisothiocyanate linking. The binding of DNA onto the films is shown to depend on films' composition having an optimum where the binding is substantially superior compared to commercial analogues. The relevant properties of the films are characterized by AFM, FTIR and MALDI-TOF MS measurements.

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1. 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.

The structure of silane layer formation on the substrate surface heavily depends on the nature of silane, i.e. length of

its molecule [2] and terminal functionality [2,3], and also on trace quantities of water in reaction medium and on the substrate [4,5]. In search for a robust procedure for reproducible fabrication of silane coatings we have proposed an alternative silanization technique that was less dependent on humidity and nature of the substrate and enabled to prepare homogeneous and smooth aminosiloxane surfaces [6]. This was achieved by dip coating of mica substrate with partially pre-polymerized (3-aminopropyl)trimethoxysilane (APTMS) sol, followed by its gelation in humid air. Still, the films did not feature prolonged stability in water, which was probably caused by low rate of cross-linking between individual siloxane molecules. In the present study we focus on fabrication of (3-aminopropyl)trimethoxysilane–tetramethoxysilane (APTMS–TMOS) hybrid films in search for new and improved substrates for potential use in DNA microarray analyses. The ability of the films to bind 25-mer oligonucleotide DNA is discussed in comparison with their commercial analogues (SAL-1 slides, Asper Biotech Ltd. [7]). The characteristics of the films are investigated by FTIR spectroscopy (Fourier transform infrared spectroscopy), MALDI-TOF mass spectrometry (matrix assisted laser desorption ionization-time of flight mass spectrometry) and AFM (atomic force microscopy) measurements.

* Corresponding author. Institute of Physics, University of Tartu, 142 Riia St., 51014 Tartu, Estonia.

E-mail address: saal@fi.tartu.ee (K. Saal).

2. Experimental

2.1. Cleaning of glass slides before silanization

In order to exclude the possible effects of impurities on reproducibility of coupling of silane and subsequently DNA to glass surface the slides were subjected to cleaning procedure developed in Asper Biotech Ltd. Glass slides ($75 \times 25 \times 1$ mm, Waldemar Knittel Glasbearbeitungs GmbH and Co KG) were sonicated for 10 min in 0.5% aqueous Alconox solution (Sigma-Aldrich Co), washed thoroughly with distilled water and sonicated for 10 min in acetone (Naxo Ltd, analytical grade). Thereafter the slides were gently shaken for 1 h in 3 M NaOH solution in 1:1 v/v mixture of water/95% ethanol (Naxo Ltd, analytical grade) and thoroughly washed with distilled water. Finally, the water was expelled by centrifugation of slides at $280 \times g$ (Jouan CR422) for 3 min and the slides were stored in clean box until usage.

2.2. Preparation of APTMS–TMOS films

APTMS and TMOS (both Sigma-Aldrich Co) were mixed at molar ratios 0:1, 1:10, 1:5, 1:3, 1:1, 3:1, 5:1, 10:1, and 1:0, respectively. Then, at room temperature and constant stirring, a mixture of water/methanol (Naxo Ltd, 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. In the case of pure TMOS (APTMS–TMOS 0:1) the mixture of water/methanol was acidified with concentrated HCl, so that the final molar ratio of TMOS/H₂O/MeOH/HCl was 1:2:2:0.005. 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.

For silanization of glass slides the stock solutions were diluted 40 times with dry methanol and subsequently the slides were dipped in these solutions. Thereafter the slides were kept in open air (relative humidity 30%) for 48 h, and subsequently the temperature was raised to 140 °C (0.3 °C/min) for 12 h.

The thickness of prepared films was estimated with AFM (see 2.5). Freshly prepared films were scraped with a pin and processed as described above (unprocessed films are soft and there is possible to avoid harming the underlying glass surface when scraping). The AFM measurements were performed at edges of the scratches. The thickness of the films was in the order of 30 nm.

2.3. Immobilization of 25-mer oligonucleotide DNA onto silanized slides and DNA spot analysis

The silanized slides were gently shaken in 0.2% w/w 1,4-phenylenediisothiocyanate (Sigma-Aldrich Co) solution in 10% w/w pyridine/dimethylformamide (Fluka, analytical grade) for 2 h, which activated the slides for immobilization of DNA. Then the slides were thoroughly washed with

acetone, methanol and ethanol (Naxo Ltd) and centrifuged at $280 \times g$ for 3 min.

1 part of Cy3 3' labelled 5'-aminomodified 25-mer oligonucleotide DNA (a type of fluorescent-labelled oligonucleotide DNA, MWG-Biotech) was mixed with 100 parts of unlabelled 25-mer oligonucleotide DNA (MWG-Biotech). The mixture was spotted to glass slides with a spotter (Virtek CWP) in 100, 80, 50, 30, 10, 3, 1, 0.4 and 0.1 μ M series, respectively. For dilutions Genorama Spotting Solution Type I (Asper Biotech Ltd.) 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 in order to block residual aminoreactive groups, washed thoroughly with hot distilled water and wiped dry by centrifugation at $280 \times g$ 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 phenylenediisothiocyanate) were processed in the similar manner.

The fluorescence of DNA spots was detected using ScanArray 5000 (Perkin-Elmer Inc.) microarray scanner. The spots were analyzed with Genorama Genotyping Software 4.0 (Asper Biotech Ltd).

2.4. Spectroscopic measurements

Chemical groups and transformations during heating process (see 2.2) on silanized slides were analyzed by FTIR spectroscopic measurements. IR spectra were measured with Perkin-Elmer PC 16 FTIR spectrometer. A conventional Perkin-Elmer equipment was used for preparation of KBr pellets (ϕ 12 mm) by compressing spectroscopically pure KBr powder under 10 tons of weight. Freshly prepared pellets were coated with solutions of pre-polymerized precursors of pure and mixed APTMS and TMOS in methanol and post-treated as described in 2.2.

MALDI TOF mass spectrometry measurements were performed with an instrument designed at the National Institute of Chemical and Biological Physics of Estonia using 1,8,9-trihydroxyanthracene (dithranol, Sigma Aldrich Co) as matrix.

2.5. AFM measurements

The topographic features of APTMS–TMOS-films were investigated with an atomic force microscope SMENA-B (NT-MDT) working in semi-contact mode in air (at 20 °C, relative humidity 30%) using ultrasharp non-contact “Golden” silicon cantilevers NSG11 (NT-MDT). Different locations typically spanning over several square cm were scanned with different resolutions on each sample for reliable characterization of a sample.

3. Results and discussion

3.1. Immobilization of DNA to APTMS–TMOS films

A series of APTMS–TMOS films were prepared by variation of the relative content of two silanes and measured their ability to bind 25-

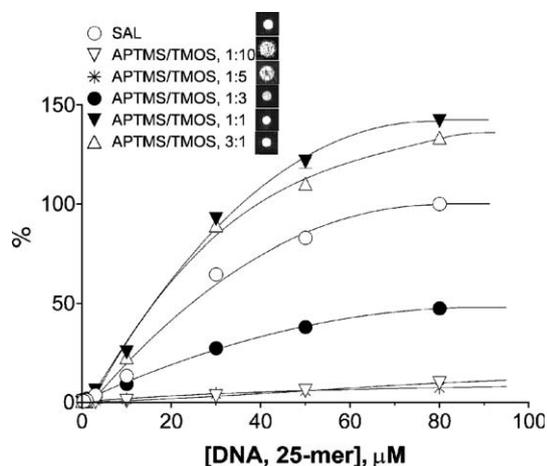


Fig. 1. The binding curves of 25-mer oligonucleotide DNA to APTMS–TMOS hybrid films, normalized to the signal of 80 μM DNA spot on the SAL-slide. Each data point corresponds to an average fluorescence intensity of 16 independent spots. The inset next to legends on the plot shows the typical fluorescence images ($300 \times 300 \mu\text{m}^2$) of the 50 μM spots on the corresponding films.

mer oligonucleotide DNA (Fig. 1). In the case of APTMS–TMOS 0:1 film no binding was detected, which was because of the absence of isothiocyanate groups on the surface. It indicated also that the non-specific binding of aminated DNA to the film was very low. The binding of DNA to APTMS–TMOS 1:10 and 1:5 films was also low remaining on the level of 10% of the commercial SAL film binding. Further increase of the content of APTMS in the mixture gave considerable rise in the amount of immobilised DNA, and with the equimolar mixture the signal achieved 140% level of the SAL-glass (Fig. 1). Similar high binding was achieved also in the case of APTMS–TMOS 3:1 films, but increase of APTMS excess to 5 fold or higher led to diffuse DNA spots, which binding efficiencies could not be reliably obtained. The latter conforms with expectations that low fraction of TMOS in the mixture causes slower and lower cross-linking between aminosiloxane oligomers and therefore the formed film is not stable in aqueous solutions.

It is important to note that the dimensions of the DNA spots decreased with the increase of the amount of APTMS used for the films (Fig. 1., inset). The size of the spots correlates with the wettability of the surface, which is determined by the amount of hydrophobic aminopropyl groups. Therefore, for the best practical conditions—

maximal fluorescence signal within minimal area—an optimal mixture should be selected.

Analogous aminopropyl embedded silica films were used for further studies on their potential use in DNA microarray analysis performing Arrayed Primer Extension (APEX) reactions mutation analysis [8]. Detailed description of microarray results is described in Ref. [9].

The layer of DNA on APTMS–TMOS 1:1 film was uniform showing some roughness only on nanometer scale, which allowed also clearly visualize the edge of the DNA spot (Fig. 2a). Note that smoothness of the surface, its stability and presence of sufficient reactive groups for the immobilization is a critical condition for higher resolution visualization of biomolecules. The surface of the SAL-slide had higher surface roughness both inside and outside the spot area (Fig. 2b), which could originate either from topographical features of the glass surface or immobilized siloxane clusters.

3.2. Spectroscopic data of APTMS–TMOS precursors and films

The formation of precursors and films was studied by FTIR and MALDI TOF mass spectrometry. It was observed that unbaked films had relatively strong absorption at $3342\text{--}3420 \text{ cm}^{-1}$, a band that corresponds to OH stretching of SiOH, CH_3OH and H_2O (Fig. 3). After heating at $140 \text{ }^\circ\text{C}$ the intensity of this absorption decreased substantially and starting from the APTMS–TMOS 1:1 film two well defined signals appeared at 3366 and 3284 cm^{-1} . These bands correspond to the antisymmetric and symmetric stretching of NH_2 group, respectively. Surprisingly, these two absorptions were not detected even in pure APTMS precursor. The reason could be an overlap with strong vibration of OH bond or formation of hydrogen bond between NH_2 and SiOH groups before the film formation, which is in agreement with the proposed mechanism of formation of APTMS layer on silica [10].

The most intriguing region is between 1700 and 900 cm^{-1} . The spectra of unbaked films clearly showed strong signals at 1580 cm^{-1} that corresponds to N–H scissoring vibration and at 1486 cm^{-1} that is believed to correspond to symmetric NH_3^+ deformation mode partly superimposed by CH_2 bending [11]. After heating of the precursor films the strong signal at 1486 cm^{-1} disappeared and the medium bending signal of CH_2 of the usual value of 1476 cm^{-1} was detected. The change of absorption bands corresponding to N–H and CH_2 vibrations at 1580 and 1486 cm^{-1} , respectively (Fig. 3), is supposedly caused by decomposition of the relatively labile H-bonding network between SiOH and NH_2 groups. As a result of this process the degree of polymerization increases and 3D structure is formed. At 1630 cm^{-1}

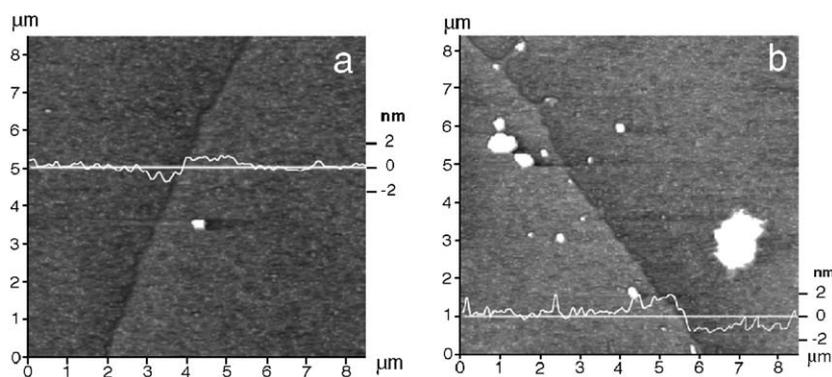


Fig. 2. Semi-contact mode AFM images of DNA spots on APTMS–TMOS 1:1 film (a) and on SAL slide (b); scan range $8.5 \times 8.5 \mu\text{m}^2$. The scale bars on the right correspond to the line profiles drawn on the images to illustrate sharp edges of DNA spots.

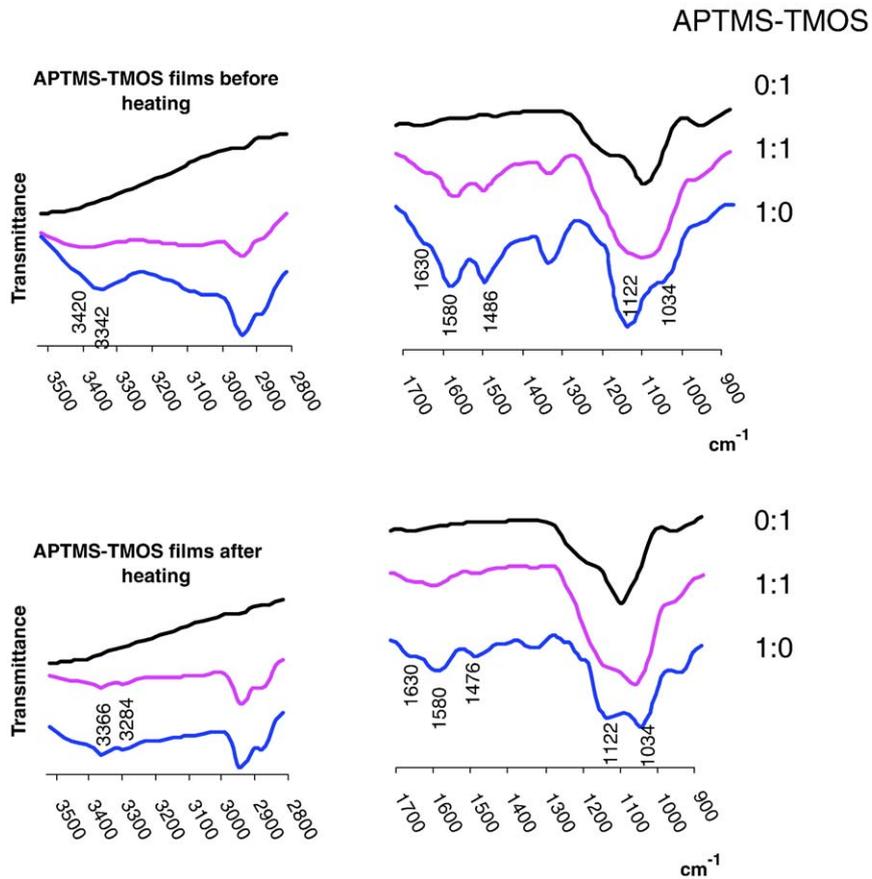


Fig. 3. FTIR spectra of APTMS–TMOS hybrid films at 3 characteristic APTMS/TMOS mixtures. The pre-polymerized APTMS–TMOS mixtures were gelled on KBr pellets and spectra recorded before and after baking the pellets at 140 °C. Several well-known absorption peaks are marked. Intensity is in arbitrary units.

only a weak shoulder in spectra of precursors as well as baked films was detected. This signal did not change during heating and we could not assign this to NH_3^+ deformation as it was proposed earlier [11].

As it can be seen in Fig. 3, two well-defined bands at 1034 and 1122 cm^{-1} appeared in the spectra of baked APTMS–TMOS films. Similar structure at 1055 and 1088 cm^{-1} in the spectra of $(\text{CH}_3)_2\text{Si}$

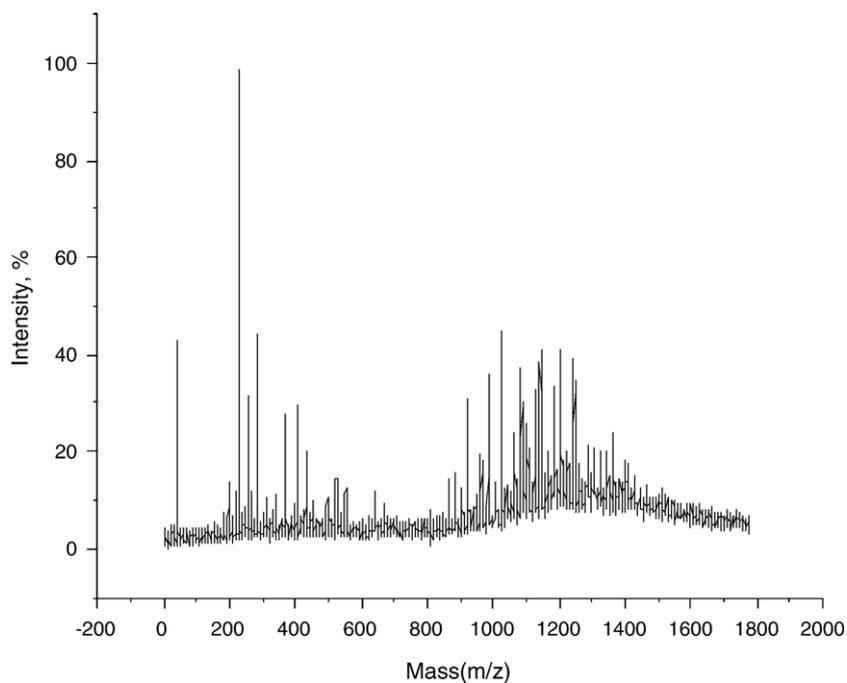


Fig. 4. MALDI TOF mass spectrum of APTMS–TMOS 1:1 hybrid material.

$(\text{OCH}_2\text{CH}_3)_2$ has been assigned to the linear and cyclic forms of siloxane polymer, respectively [[12] cited therein]. The relative shift can be also explained by structural differences of initial monomers.

MALDI TOF mass spectra revealed that APTMS–TMOS hybrid materials had molar masses in range of up to 1500 amu (Fig. 4). The mass range detected did not significantly depend on the relative compositions of APTMS/TMOS in the mixtures. As expected, the spectra had very complicated structure that contained different “families” of oligomers. For pure pre-polymerized APTMS it was estimated that such mass distribution corresponded to the oligomers containing approximately up to 12 monomers.

3.3. Surface of APTMS–TMOS films

APTMS–TMOS 0:1 film exhibited a uniform and smooth surface (average vertical difference 5 nm per 1 μm scan) (Fig. 5a). APTMS/TMOS 1:10 film showed surface consisting of grains with several to a hundred nanometers in diameter and average height distribution of

20 nm/ μm (Fig. 5b). The surfaces of APTMS/TMOS 1:5 and 1:3 films were similar to 1:10 film, but the surface line profiles indicated to substantially lower deviations in height (Fig. 5c and d). Starting from APTMS/TMOS 1:1 film the surface profiles ranged between two nanometers, thus showing practically featureless topography in 1 μm^2 scale (Fig. 5e–i).

It has been shown that in basic medium alkoxy silanes polymerise to three-dimensional nanosized siloxane particles, whereas in acidic medium linear molecules are formed [13]. This result is confirmed since the polymerisation of TMOS was carried out in acidic medium where spinnable viscous material was formed and the corresponding film showed relatively uniform surface with no evidence of grainy texture (Fig. 5a). When no acid as catalyst was used white powder-like material precipitated, indicating to the formation of branched siloxane particles. On the other hand, the polymerisation of the mixture of APTMS/TMOS can be considered as base-catalysed process because of amino groups of APTMS. Thus, in great excess of TMOS silane polymerises to nanosized granules that are clearly evident in Fig. 5b–d.

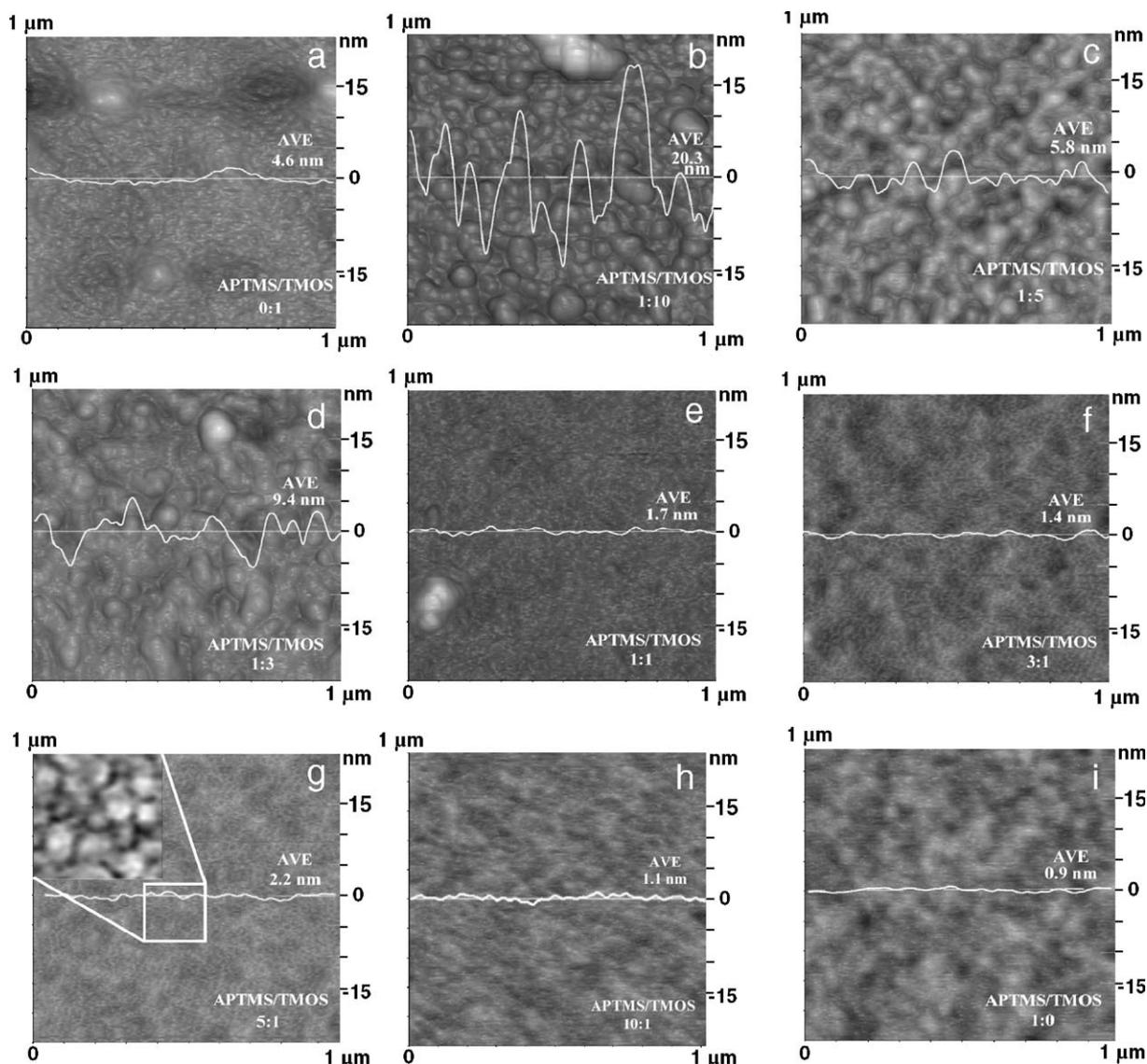


Fig. 5. Semi-contact mode AFM images of APTMS–TMOS hybrid films; scan range 1 \times 1 μm^2 . The scale bar on the right corresponds to the line profiles drawn in the middle of the scan. AVE represents the average height difference in scan profile. High-resolution inset (200 \times 200 nm^2) in (g) illustrates granule-like structure of the surface (see text).

At higher APTMS content the granules appear smaller probably because of the steric hindrance of aminopropyl groups that diminishes the condensation to progress in all directions (see inset in Fig. 5g). In between these extremes of APTMS/TMOS ratios observed we believe this steric hindrance to remain sufficient for disabling the growth of siloxane particles big enough to precipitate, which makes them use of as precursors for film making. Consequently, the APTMS/TMOS hybrid films can be thought of as consisting of densely packed nanosized siloxane particles, and the dimensions as well as the rate of cross-linking between individual “building blocks” are determined by the ratio of APTMS/TMOS.

4. Conclusions

1. It was shown that APTMS–TMOS hybrid films have potential as substrates for immobilisation of aminated DNA via 1,4-phenylenediisothiocyanate linking.
2. The ratio of APTMS to TMOS chosen in precursor synthesis determines the number of amino groups on the films’ surfaces available for immobilization of DNA and shape of the formed spots. The maximal binding of DNA was achieved on the APTMS–TMOS 1:1 and 3:1 films.
3. Films with higher APTMS molar content (starting from APTMS–TMOS 5:1 film) were not stable in aqueous medium. The instability was probably caused by relatively low content of cross-linking agent TMOS.

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