Optical characterization of aminosilane-modified silicon dioxide surface for biosensing

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Silicon dioxide surfaces, functionalized by two aminosilane compounds (3-amino-propyl-triethoxysilane, APTES; 3-amino-propyl-dimethyl-ethoxysilane, APDMES) both dissolved in different solvents (dry ethanol and toluene), have been investigated by standard techniques such as spectroscopic ellipsometry (SE), water contact angle (WCA), and atomic force microscopy (AFM). Silane thicknesses between 5 and 80 Å have been found, depending on deposition conditions; surface wettabilities change, accordingly. These organic-inorganic interfaces have also been modified by a cross-linker (bis-sulfosuccinimidyl suberate) in order to covalently bind a fluorescein labeled protein A. The amount of protein linked to functional surfaces has been quantified by SE and fluorescence microscopy. These results could be very useful in developing new platforms for optical biosensing.
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Keywords: Surface functionalization, biomaterials, ellipsometry, water contact angle, atomic force microscopy

1 INTRODUCTION

Bioconjugation chemistry is a key issue not only for fabrication of sensitive and selective biosensors, but also for many technological devices, which can be used in biomedical diagnostics and even in fundamental scientific studies. In the last few years, multifunctional lab-on-chip platforms for biomolecular interactions monitoring have been proposed [1]–[3], as well as new single molecule spectroscopy methodologies [4, 5], all based on specific passivation protocols of support surfaces. The aim of these treatments is immobilization on a solid support of biological molecules, preserving their specific functionalities through a good control of their orientation and organization. Even if glass [6] and gold [7] have been classically used, and therefore their passivation chemistries deeply studied in these kinds of application, on the other hand, silicon, and silicon related materials, are attracting growing interest, due to widespread diffusion of microfabrication technologies, well developed in the frame of consumer electronics. The most common route of silicon surfaces functionalization is to attach alkylsilanes layers through the formation of Si-O-Si bonds between the silanol groups present on oxidized silicon surface and the hydrolyzed organosilane molecules [8]. Recently, the interaction mechanism between silane layers and silicon surfaces have been deeply characterized, up to molecular level [9]–[11]. Although wet deposition by solution immersion is the most common method to prepare these samples, monofunctional and tri-functional aminosilane molecules, like 3-amino-propyl-triethoxysilane, APTES, and 3-amino-propyl-dimethyl-ethoxysilane, APDMES, respectively have been deposited on dehydrated silicon support also by chemical vapour deposition, which is a robust process, currently used in semiconductors industry [12]. APTES and APDMES have structural differences, as schematized in Figure 1. APTES has three attachment points to the surface or other silane molecules, therefore it can polymerize. Conversely, APDMES has only one attachment point and it cannot polymerize. In this work, we have experimentally characterized amino-

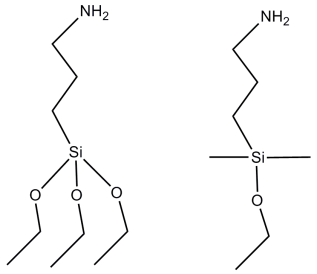

FIG. 1 Structures of 3-aminopropyltriethoxysilane (APTES) and 3-aminopropyl-dimethylethoxysilane (APDMES).

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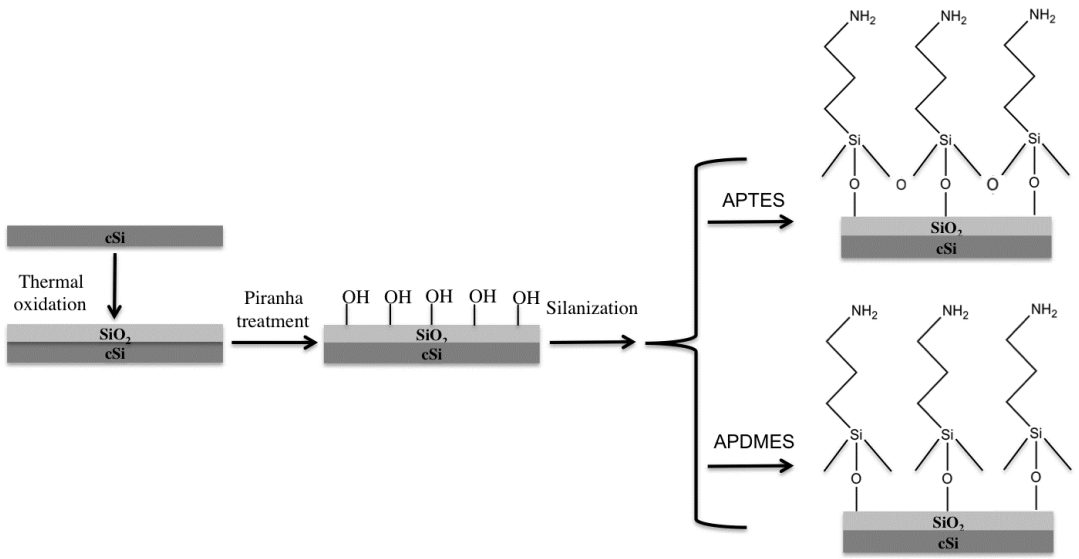

FIG. 2 Scheme of silicon surface modification by APTES and APDMES.

Sample	Silane	Solvent	Incubation time (min)
S1	Aptes 5%	Ethanol	30
S2	Aptes 5%	Ethanol	60
S3	Aptes 5%	Toluene	30
S4	Aptes 5%	Toluene	60
S5	Apdmes 5%	Ethanol	30
S6	Apdmes 5%	Ethanol	60
S7	Apdmes 5%	Toluene	30
S8	Apdmes 5%	Toluene	60

TABLE 1 Sample preparation conditions.

modified silicon dioxide surfaces by several techniques, such as spectroscopic ellipsometry (SE), water contact angle (WCA), and atomic force microscopy (AFM). Data highlight some important features of these surface passivation strategies useful in realization of immune-arrays or, in general, bioconjugated devices.

2 MATERIAL AND METHODS

2.1 Silane surface modifications

Highly doped p^+ silicon wafer, <100> oriented, 0.003 Ω·cm resistivity, 400 μm thick, was cut into 10 mm × 10 mm square pieces. After cleaning by means of standard RCA process [13], silicon substrates were thermally oxidized at 1050°C for 5 hours. Chips were, then, immersed in piranha solution (H_2SO_4:H_2O_2=4:1) at room temperature for 30 min so as to create Si-OH groups on silicon surface, extensively washed in milli Q water, and dried in a stream of nitrogen gas. Eight different silane films, namely S1-S8, were obtained by incubating the silicon substrates at room temperature, for 30 or 60 min, into 5% silane solutions prepared by direct dissolution of silane, APTES or APDMES, in ethanol or anhydrous toluene, as summarized in Table 1. After silanization, silicon chips were rinsed three times in the solvent used for the process for 2 min so as to remove silane excess. The last step is silane curing on heater at 100°C for 10 min. The scheme of silanization process performed on silicon surface is reported in Figure 2. The experiment has been performed on two sets of identical samples to confirm results.

2.2 Biofunctionalization

Chemicals and solvents were purchased from Sigma-Aldrich. Protein A labeled with -FITC (PrA*) was immobilized on silane modified silicon surface using bis(sulfosuccinimidyl)suberate (BS^3) crosslinker. The scheme of functionalization process is reported in Figure 3. Each chip was incubated with 150 μl of 1.6 mM BS^3 in PBS solution (0.1 M; pH=7.4) at 4°C for 5 hours. N-hydroxysulfosuccinimide (NHS) ester reacts (through SN_2) with primary amines of silanized surface forming stable amine bonds and releasing a NHS group. The functionalized substrate was then incubated overnight (ON) at 4°C with 150 μl of 2 mg/ml PrA* in PBS (0.1 M; pH=7.4) buffer. NHS ester reacts with primary amines in the side chain of lysine residues of PrA* forming stable amine bonds and releasing an other one NHS group.

2.3 Spectroscopic ellipsometry

Spectroscopic ellipsometry (SE) measurements were performed by a Jobin Yvon UVISEL-NIR phase modulated spectroscopic ellipsometer apparatus, at an angle of incidence of 65°over the range 300–1600 nm with a resolution of 5 nm. The instrument measures the spectral variation of the ellipsometric angles Ψ and Δ defined through the relation:

$$tg\Psi e^{i\Delta} = \frac{R_p}{R_s} \quad (1)$$

where R_p and R_s are the complex reflection coefficients of the light polarized parallel and perpendicular to plane of incidence. Thickness of films present on silicon surface was determined from the ellipsometric data analysis using Delta Psi software [14].

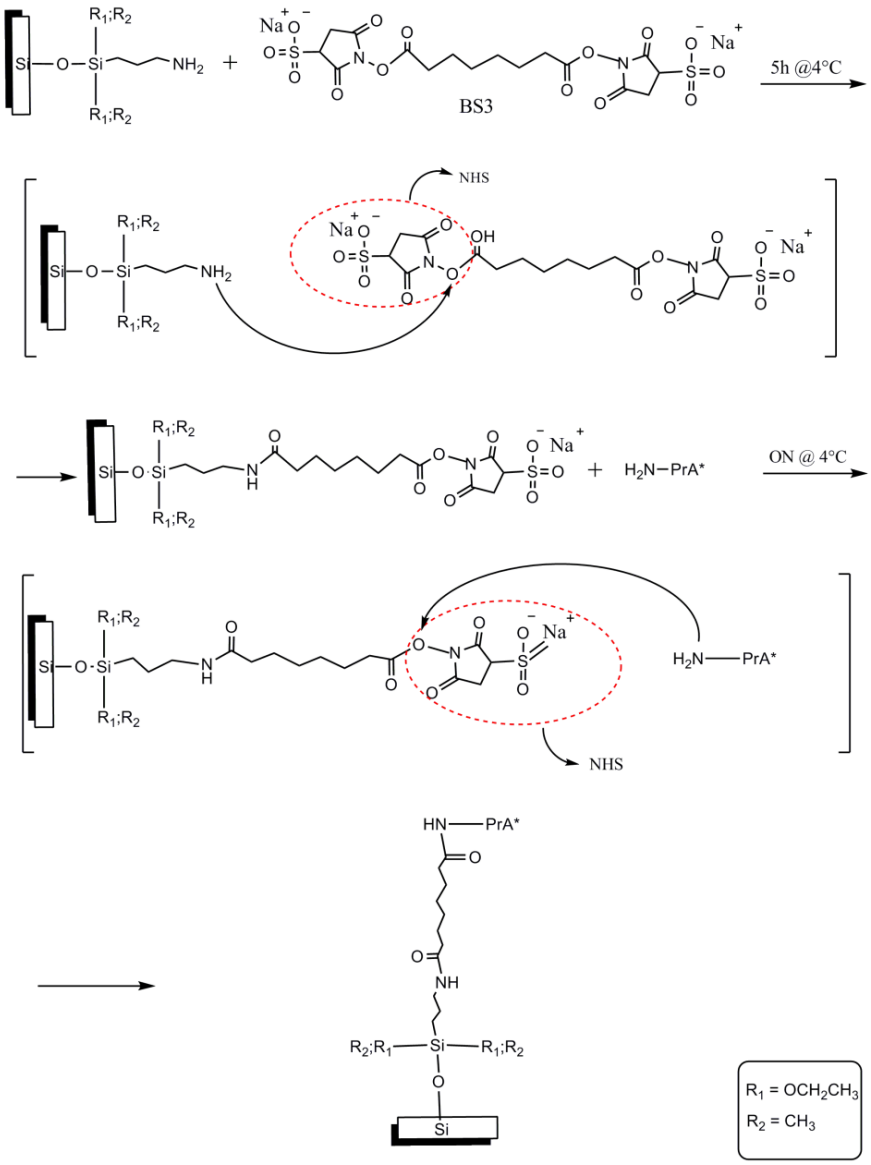

FIG. 3 Functionalization of silane modified cSi surface with PrA*.

2.4 Water contact angle measurements

Sessile drop technique has been used for water contact angle (WCA) measurements on a First Ten Angstroms FTA 1000 C Class coupled with drop shape analysis software. The WCA values reported in this work are the average of at least three measurements.

2.5 Atomic Force Microscopy

A XE-100 AFM (Park Systems) was used for the imaging of biofilms. Surface imaging was obtained in non-contact mode using Silicon/aluminum coated cantilevers (PPP-NCHR 10M; Park Systems) 125 μm long with a resonance frequency of 200 to 400 kHz and nominal force constant of 42 N/m. The scan frequency was typically 1 Hz per line. Roughness has been calculated on 3 μm × 3 μm images.

2.6 Fluorescence microscopy

Fluorescence analysis was performed by means of a Leica Z16 APO fluorescence macroscope equipped with a camera Leica DFC300. I3 filter was used for image acquisition consists in a 450–490 nm band-pass excitation filter, a 510 nm dichromatic mirror, and a 515 nm suppression filter. Fluorescence intensity values reported in the work are averaged on three measurements.

3 RESULTS AND DISCUSSION

A precise estimation of silane layers thickness requires the ellipsometric analysis of samples before and after silanization processes. The measure performed on bare silicon dioxide (before silanization) allows an exact determination of its thickness (95.46 ± 0.03 nm) and a consequence certain estimation of silane layer. Refractive index of silicon dioxide as function of wavelength is taken from reference [15].

Spectroscopic ellipsometry data, reported in Table 2 together with water contact angle variations (i.e., the difference between the WCA of sample after and before silanization process. WCA of bare silicon dioxide surface is (60.8 ± 0.8)°), reveal a common trend for all characterized samples: the esti-

Silane	n_{silane}	d (g/cm^3)	M (g/mol)	V_m (cm^3/mol)	A (cm^3/mol)
APTES	1.46	0.946	221.37	234	65
APDMES	1.46	0.857	161.32	188	52

TABLE 3 Ellipsometry data and increase of WCA after silanization processes.

Sample	Silane thickness (Å)	ΔWCA (°)
S1	5.5±0.3	3±1
S2	17.3±0.3	12.3±0.8
S3	26.4±0.3	7.2±0.6
S4	81.3±0.8	21±3
S5	0.2±0.2	2±1
S6	3.0±0.3	10±3
S7	4.2±0.3	19±3
S8	10.6±0.3	15±2

TABLE 2 Ellipsometry data and increase of WCA after silanization processes.

Sample	M/A (g/cm^3)	Γ (μg/cm^3)
S1	3.406	0.0051±0.0003
S2	3.406	0.0161±0.0003
S3	3.406	0.0246±0.0003
S4	3.406	0.0758±0.0007
S5	3.102	0.0001±0.0002
S6	3.102	0.0025±0.0003
S7	3.102	0.0036±0.0003
S8	3.102	0.0090±0.0003

TABLE 4 Surface concentration, Γ.

Sample	BS^3 thickness Å	PrA* thickness Å
S1	7.5±0.0.3	14.5±0.4
S2	22.9±0.3	9.4±0.4
S3	9.3±0.3	22.3±0.5
S4	18.9±0.8	7.9±0.8
S5	5.6±0.3	10.2±0.3
S6	13.1±0.3	8.5±0.4
S7	10.0±0.3	10.1±0.4
S8	5.7±0.3	11.1±0.4

TABLE 5 Thicknesses of organic and biological layers, respectively BS^3 and PrA*, linked to silane modified cSi surface. The values have been obtained by means of ellipsometric characterization.

mated siloxanes film thickness increases with time deposition: 60 min samples are always thicker than 30 min samples in every combination of solute (APTES and APDMES) and solvent (ethanol and toluene). Moreover, APTES thin films are thicker than APDMES ones: a somehow expected result since APTES is able to reticulate, while APDMES is not. WCA measurements highlight that both APTES and APDMES surfaces can be sensitively less hydrophilic than uncovered ones. However, WCA values are scattered; this behavior can be explained considering film inhomogeneity due to the deposition by solution. Another very interesting application of spectroscopic ellipsometry is calculation of surface concentration that gives idea of how dense is the film deposited on the surface. To this aim, molar refractivity A of a material can be defined by the formula [16]:

$$A = \frac{(n^2-1)}{(n^2+2)} V_m \tag{2}$$

where n is the refractive index, and V_m the molar volume calculated as the ratio M/d, with M molecular weight, and d density. Estimated values of molar refractivity for APTES and APDMES have been reported in Table 3 together with parameters used to calculate them; refractive indexes n were determined by means of spectroscopic ellipsometry using a fixed index model (i.e., n(λ)=constant) [11]; molecular weight M, and density d were taken from data sheet of chemical compounds.

These values were used to calculate surface concentration Γ (μg/cm^2) of silane layers deposited on silicon chips, by Cuypers formula [17]:

$$\Gamma = 0.1t \frac{M}{A} \frac{n_f^2-1}{n_f^2+2} \tag{3}$$

where thickness t has been calculated by SE (see values in Table 2). In Table 4, we have reported the ratio M/A used to calculate Γ, and values of Γ.

Just like film thicknesses, also the surface concentrations follow the same behavior: as longer time deposition is, greater is the value of Γ. We have repeated SE characterization after incubation with BS^3 cross-linker and with PrA*. Refractive indexes used for BS^3-cross linker and PrA* are 1.43 and 1.46, respectively. BS^3 refractive index is reported by reference [18]. PrA* refractive index has been calculated from Eq. (2) using the following values [19]: molar refractivity, A=10173 cm^3/mol; molar volume, V_m=37180 cm^3/mol (V_m is the product of molecular weight, M_w=41000 g/mol times the partial specific volume, V_p=0.715 cm^3/g). Results of ellipsometric characterization are reported in Table 5.

The thickness of organic and biological films ranges between about 5 and 23 Å and it does not seem to be correlated to silane thickness. From SE data, we can infer that more protein A can be bound to the surface in case of 30 min silanized chip (namely S1, S3, S5, S7) since the PrA* estimated thickness is always greater than BS^3 layer thickness. When there is too much cross-linker, less protein can be found, probably due to steric hindrance of molecules (see values for S2, S4, S6). These considerations should be carefully trusted, since SE only gives a macroscopic average of layers thickness on an area of 1 mm^2, which is not directly correlated to nanometric size of deposited films. We can certainly conclude that both organic and biological layers are of the same thickness order and

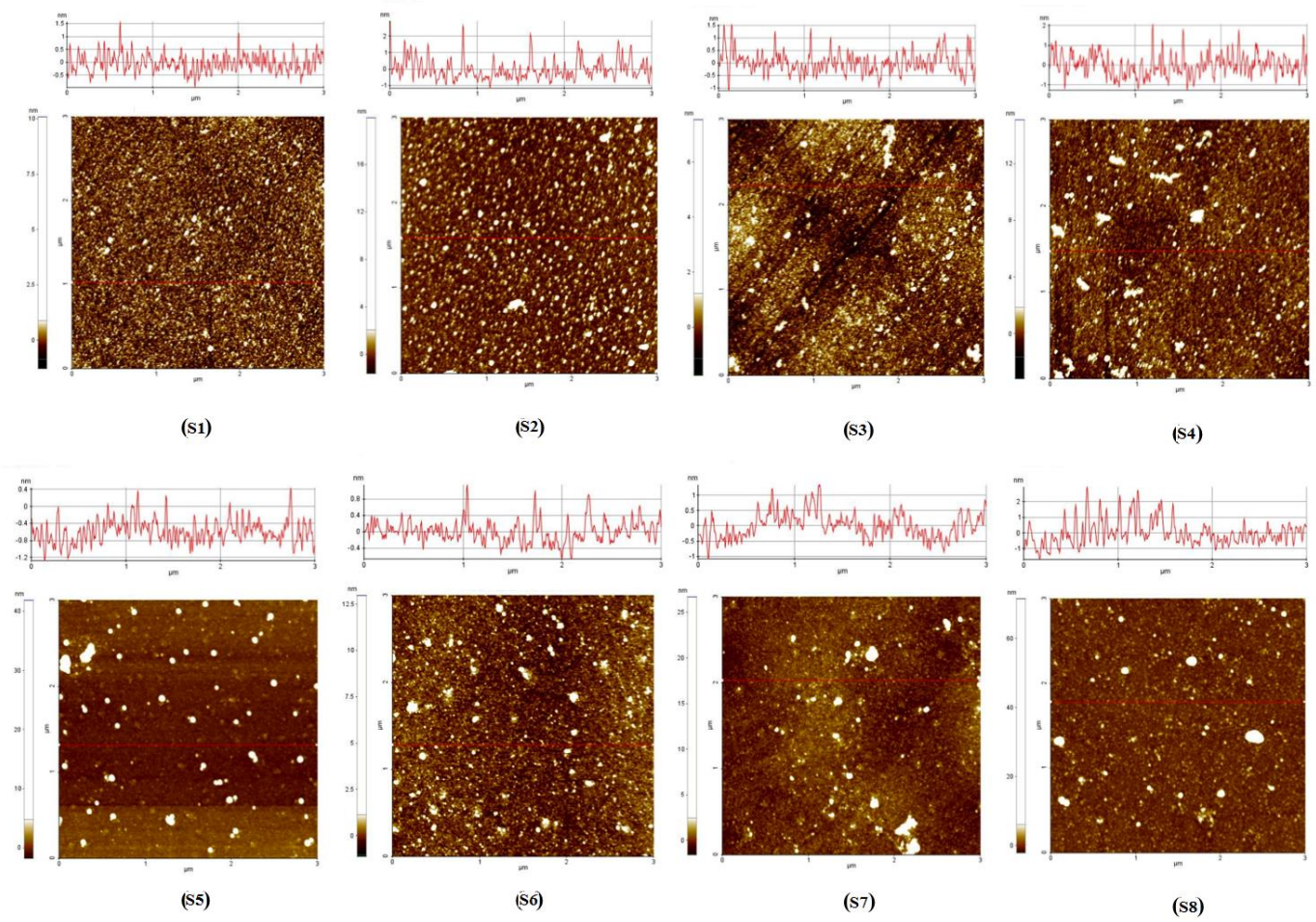

FIG. 5 3 µm wide AFM images of aminosilane modified CSi surfaces after PrA* functionalization.

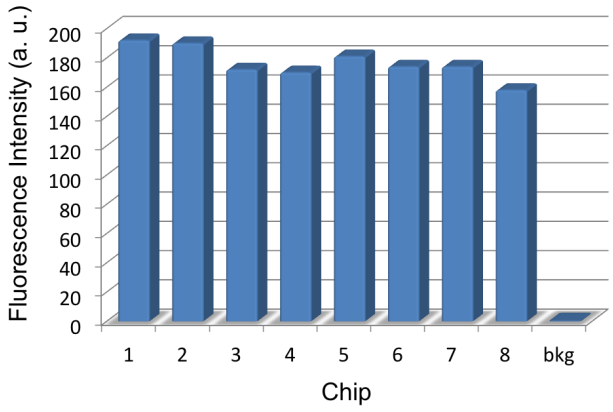

FIG. 4 Fluorescence intensity measured on the chips functionalized with PrA* and on background (silane modified cSi surface + BS^3).

Sample	Fluorescence intensity (a. u.)
S1	192±2
S2	190±6
S3	172±1
S4	170±5
S5	181±2
S6	174±2
S7	174±2
S8	158±3

TABLE 6 Fluorescence intensity measured on the chips functionalized with PrA*. The values have been obtained as average of three different measurements on the same sample.

that less cross-linker is better than too much. This conclusion is also supported by fluorescence data (Figure 4), summarized in details in Table 6. Fluorescence intensity is an integrated density calculated on an area of 100 × 100 μm^2 using a free-ware software, ImageJ. Each value reported in Table 6 is the average of three independent measurements on same sample. Fluorescence intensity of bare silicon dioxide is (7±2) a. u.

The fluorescence intensity of odd samples (which has been incubated for 30 min) is always greater than that of even ones (incubated 60 min), independently of considered silane. Anyway, absolute values of fluorescent intensities are very close each other, so that all samples have been effectively functionalized.

Quite differently, AFM images and characterization highlight some distinctions between PrA* surfaces on APTES and APDMES modified supports (see Figure 5). Quantitative measurements are expressed in terms of roughness, which values are reported for all samples in Table 7. Roughness of thermally grown silicon dioxide is 0.250 nm.

Sample	Roughness (nm)
S1	0.353
S2	0.605
S3	0.416
S4	0.550
S5	0.264
S6	0.294
S7	0.353
S8	0.776

TABLE 7 Root mean square roughness values of sample surfaces after PrA* functionalization measured using an AFM ($3 \times 3\ \mu m^3$)

By examining both series (S1-S4; S5-S8) of number, it is evident that APDMES samples are smoother than APTES ones, except for sample S8 that is quite always very different from S5-S7. Moreover, in case of APTES samples, the thinner is the protein layer, the greater is the roughness, probably because of vertical inhomogeneity of APTES layer and PrA* film are very similar. APTES treated surfaces seem more crowded than APDMES ones, and again we believe that this is also due to self-assembling nature of APTES with respect to APDMES.

4 CONCLUSIONS

We have successfully functionalized silane modified flat oxidized silicon surfaces by ordinary chemical procedure, and we have studied how different solvents and incubation times can affect the quality of the protein layer on top. Quantitative measurements based on SE, WCA and AFM reveal that smoother and homogeneous film can be obtained using APDMES in toluene incubated for 30 min. Anyway, all other samples show good functionalization degree as it can be seen by fluorescence characterization.

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