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Aminosilane functionalizations of mesoporous oxidized silicon for oligonucleotide synthesis and detection

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Direct solid phase synthesis of peptides and oligonucleotides (ONs) requires high chemical stability of the support material. In this work, we have investigated the passivation ability of porous oxidized silicon multilayered structures by two aminosilane compounds, 3-aminopropyltriethoxysilane and 3-aminopropyltrimethylethoxysilane (APDMES), for optical label-free ON biosensor fabrication. We have also studied by spectroscopic reflectometry the hybridization between a 13 bases ON, directly grown on the aminosilane modified porous oxidized silicon by *in situ* synthesis, and its complementary sequence. Even if the results show that both devices are stable to the chemicals (carbonate/methanol) used, the porous silica structure passivated by APDMES reveals higher functionalization degree due to less steric hindrance of pores.

1. Introduction

Fabrication of biosensors and microarrays, and more generally of an inorganic device coupled to a biomolecule, has its main issue in bioconjugation, i.e. the immobilization of a working biological probe onto a solid surface. Bioconjugation represents a current challenge crossing both material sciences and biomedical applications [1–3]. Lots of chemical protocols have been reported in the literature in order to passivate and functionalize surfaces: the immobilization of biological species should not prevent their functions, and thus should also ensure their correct organization and orientation. The standard example in this field is gold functionalization by self-assembled monolayer, which gives an appropriate distance from the support surface and a strong binding to biomolecules. In the case of semiconductors and their related materials (oxides, nitrides and their porous formulae), it is not so straightforward to find a common route of functionalization: in particular, on silicon and silicon dioxide surfaces, organosilane reagents are commonly used to obtain passivation layers that can be successfully employed to attach protein or other biomolecules in an easy way [4,5]. The alkylsilanes, like 3-aminopropyltriethoxysilane (APTES) and 3-aminopropyltrimethylethoxysilane (APDMES), can be linked to a hydrolysed silicon surface through the formation of Si–O–Si bonds. The reaction is not trivial, and the quality of the interface is not always the same, depending on the silane and the procedure conditions [6]. Recently, we have demonstrated *in situ* synthesis of oligonucleotides (ONs) on the surface of porous silica structures, characterized by optical monitoring [7,8]; the advantages of *in situ* synthesis with respect to *ex situ* immobilization are not only the increasing of DNA probe density but also the process automation, and the possibility of surface local functionalization [9]. Porous silicon (PSi) is obtained by electrochemical partial dissolution of crystalline silicon in a hydrofluoric solution, and therefore exhibits a sponge-like morphology, characterized by very high specific

surface, up to hundreds of $\text{m}^2 \text{cm}^{-3}$. Moreover, PSi is by far one of the most intriguing materials in optical sensing: the refractive index is widely tuneable, namely between the silicon refractive index and that of air, and it is changed by each substance that penetrates into its pores. Owing to these characteristics, a lot of optical structures, such as photonic filters and microcavities, have been proposed in the literature for chemical [10] and biological sensing [9]. Unfortunately, the PSi structures suffer instability from oxidation and corrosion in aqueous solutions, especially simulating biological conditions. Many strategies have been developed to stabilize PSi for applications in the fields of biotechnology and biosensing [11–13]. In this work, we have studied the stabilization of oxidized PSi multilayers functionalized by APTES and APDMES, and compared the effectiveness for *in situ* ON synthesis and hybridization. Our findings demonstrate that even if APDMES forms a thinner silane layer with respect to APTES, it imparts good chemical stability to PSi support on exposure to corrosive environments and gives greater functionalization coverage.

2. Experimental

2.1. Porous silicon microcavity fabrication

We have realized a twin pair of PSi microcavities constituted by a $\lambda/2$ layer (optical thickness) sandwiched between two 9.5 period Bragg reflectors (BRs), obtaining alternating low (L) and high (H) refractive index layers. The sequence of the layers is: LHLHLHLHLHLHLHL(HH)LHLHLHLHLHLHLHLHLHL. The thicknesses, d_L and d_H , of the layers constituting the BRs satisfy the relation $n_H d_H + n_L d_L = m\lambda_B/2$ with n_H and n_L being high and low refractive indexes, m an integer and λ_B the Bragg wavelength. The microcavities were fabricated by electrochemical etching of p^+ crystalline silicon ($0.001 \Omega \text{ cm}$ resistivity, $\langle 100 \rangle$ oriented, $500 \mu\text{m}$ thick) in hydrofluoric acid (HF; 50% in weight): ethanol = 1 : 1 solution in dark and at room temperature (RT). Before the anodization process, the silicon substrate was immersed in HF solution for 2 min so as to remove the native oxide layer. The low refractive index layers (high porosity) were obtained applying a current density of 200 mA cm^{-2} for 1.2 s ($n_L = 1.542$; $d_L = 125 \text{ nm}$); the high refractive index layers (low porosity) applying a current density of 100 mA cm^{-2} for 1.4 s ($n_H = 1.784$; $d_H = 108 \text{ nm}$).

After the electrochemical process, pore dimension was increased so as to favour the infiltration of biological matter by rinsing the 'as-etched' PSi microcavities in a KOH–ethanol solution (1.5 mM) for 15 min [14]. The devices were then thermally oxidized against uncontrolled environmental ageing and corrosion in alkaline solutions. The oxidation was performed in pure O_2 applying a two-step process: 30 min at 400°C followed by 15 min at 900°C .

2.2. Surface modification with aminosilanes

The oxidized PSi microcavities were immersed in Piranha solution ($\text{H}_2\text{SO}_4 : \text{H}_2\text{O}_2 = 4 : 1$) for 40 min at RT so as to generate Si–OH bonds on their surfaces. After Piranha treatment, samples were rinsed in deionized water and dried by nitrogen gas. The structures were then silanized (functionalization by aminosilane: APTES and APDMES) by immersion at RT. The reaction conditions were optimized on crystalline silicon

varying solvent (ethanol and toluene) for silane dissolution and incubation time (30 and 60 min); thickness and uniformity of APTES and APDMES layers were investigated by spectroscopic ellipsometry (data not shown here). Best results were obtained for samples incubated in solutions containing 5 per cent aminosilane in dry toluene for 30 min. These conditions were used to graft the silane molecules on the PSi surfaces; excess of ungrafted silanes was removed by rinsing samples three times in dry toluene for 2 min. Last step of silanization process was curing on a heater at 100°C for 10 min.

2.3. Oligonucleotide synthesis

Chemicals and solvents were purchased from Fluka-Sigma-Aldrich, controlled pore glass (CPG) 1-(4,4'-dimethoxytrityloxy)-2-(6-carboxy-(di-O-pivaloil-fluorescein)-4-aminobutyl)-propyl-3-O-succinoyl-long-chain alkylamino-CPG (Fluo-CPG) was purchased from Link Technologies. Reagents and nucleoside-3'-phosphoramidites for ON synthesis were purchased from Glen Research. Solid phase ON syntheses were performed on a PerSeptive Biosystems Expedite 8909 DNA automated synthesizer. The reaction scheme of the ON synthesis on the PSi platform is reported in figure 1.

The aminosilane modified structure ($\text{PSi-L}_{a,b}\text{-NH}_2$, where $\text{L}_a = \text{APTES}$ and $\text{L}_b = \text{APDMES}$) was introduced in a suitable column reactor before synthesis. The 13 bases oligomer was assembled on the chip following phosphoramidite chemistry summarized in figure 1 and described in detail in the study of Rea *et al.* [8], thus obtaining the $\text{PSi-L}_{a,b}\text{-T}_{13}$ functionalized surface. Labelled complementary and non-complementary targets (Fluo-dA₂₀ and Fluo-dC₂₀) were prepared by solid phase synthetic methodology using 3'-phosphoramidite standard procedure [15,16] starting from Fluo-CPG by 20 cycles of growth with D-adenosine-3'-phosphoramidite or D-citidine-3'-phosphoramidite. In particular, after automated synthesis, the ONs were detached from the support and deprotected by using concentrated aqueous ammonia (33%) at 55°C for 17 h. The combined filtrates and washings were dried, redissolved in water, analysed and purified by high-performance liquid chromatography (HPLC) on an anion exchange column (Macherey–Nagel, 1000–8/46, $4.4 \times 50 \text{ mm}$, $5 \mu\text{m}$) using a linear gradient from 100 per cent buffer A (0% buffer B) to 100 per cent buffer B (0% buffer A) in 30 min (flow rate = 1 ml min^{-1} ; detection at 260 nm), where buffer A is 20 mM NaH_2PO_4 aqueous solution, pH 7.0, containing 20% (v/v) CH_3CN , and buffer B is 20 mM NaH_2PO_4 aqueous solution, pH 7.0, containing 1 M NaCl and 20% (v/v) CH_3CN . After HPLC purification, the ON samples were desalted on a Biorad P2-biogel column eluted with H_2O /ethanol (9 : 1 v/v).

2.4. Oligonucleotide phosphates-deprotection and hybridization

Hybridization with a complementary target requires the phosphates-deprotection of each base constituting the ON grown on the PSi [15]. We have previously verified, by optical measurements, that standard deprotection conditions (exposure to concentrated aqueous ammonia (33%) at 55°C for 17 h) completely corrode the PSi platform (data not shown here), therefore weak chemical conditions were used. $\text{PSi-L}_{a,b}\text{-T}_{13}$ samples were treated in 10 ml anhydrous K_2CO_3 (0.05 M)/dry methanol solution for 30 min: the protecting groups (beta-cyanoethyl) were thus removed

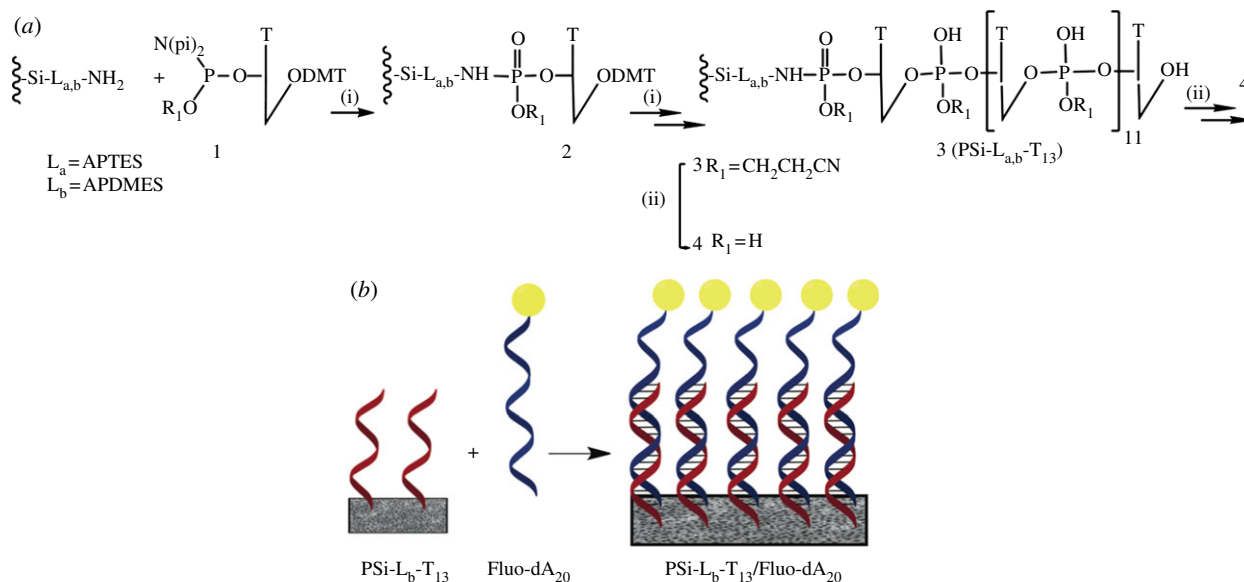


Figure 1. (a) Synthetic procedure for solid phase synthesis of aminosilane PSi-T₁₃ (PSi-L_{a,b}-T₁₃): (i) standard automatic synthetic cycle; (ii) phosphates-deprotection, K₂CO₃ (0.05 M)/MeOH dry. (b) Hybridization of aminosilane PSi-L_b-T₁₃ with Fluo-dA₂₀. (Online version in colour.)

allowing hybridization with complementary sequences (step (ii) in figure 1). The APDMES silanized structure was then cut in two pieces: one half was exposed to 300 nmol Fluo-dA₂₀ and the other one to Fluo-dC₂₀ DNA targets in 300 µl buffer solution (KH₂PO₄ 1 mM + KCl 9 mM) for 2 h.

2.5. Spectroscopic reflectometry

The reflectivity spectra of the PSi optical structures were acquired using a simple experimental set-up: a white light was sent on the PSi samples by means of a Y optical reflection probe (Avantes). The same probe was used to guide the output signal to an optical spectrum analyser (Ando AQ6315A). The spectra were acquired at normal incidence over the range 600–1200 nm with a resolution of 0.2 nm. At least three measurements were recorded so as to determine an average spectrum of the sample.

2.6. Water contact angle measurements

Sessile drop technique was used for water contact angle (WCA) measurements using 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 on the same sample.

2.7. Fluorescence microscopy

Fluorescence images were acquired by means of a Leica MZ16 FA fluorescence stereomicroscope equipped with a Leica DFC320 camera. The filter used for the acquisition was GFP2 consisting of a 460–40 nm band-pass excitation filter and a 510 nm barrier filter.

3. Results and discussion

In our previous work [8], we demonstrated the direct synthesis of a polythymine strand on nanostructured silica multilayers and analysed the process by means of a label-free technique, i.e. spectroscopic reflectometry.

Subsequently, we tried unsuccessfully the hybridization with a complementary ON target: the hydrolysed silica matrix was highly unstable and subject to corrosion not only on exposure to strong alkaline solutions such as ammonium hydroxide, generally used for phosphates and DNA bases deprotection, but also to carbonate/methanol, a weaker chemical alternatively used in deprotection processes. On the other hand, deprotection of ON phosphate groups is strictly required in hybridization with an appropriate target. Silane passivation of porous silica multilayer is the strategy to overcome the above-described intrinsic limitation of PSi scaffold. Unfortunately, both APTES and APDMES functionalized PSi microcavities are much damaged on exposure to ammonia solution (30 min), but, on the contrary, they show high chemical resistance to carbonate/dry methanol up to 2 h: in the first case, the samples are macroscopically etched; in the latter, optical spectra do not change after the chemical treatment (data not shown here).

Carbonate/dry methanol solutions are also able to remove the protecting groups (phenoxyacetyl, 4-isopropylphenoxyacetyl and acetyl groups) of the so-called ‘ultramild’ phosphoramidites, thus also allowing the synthesis of ONs having different nucleobase composition [17].

As already demonstrated in Rea *et al.* [8], thermal oxidation substitutes silicon by silica. This process is reflected in a blue shift (≈ 70 nm) of PSi multilayer reflectivity spectra, as silica has a lower value of refractive index (1.4 compared with 3.4 of silicon). Thermal oxidation also causes a decrease of pore diameter (D) of about 30 per cent, from 23 to 15 nm: the phenomenon is due to the isotropic silica expansion [18]. Thermally oxidized PSi structures were then silanized.

In figure 2, we show reflectivity spectra of PSi microcavities before (i.e. PSi multilayers thermally oxidized) and after silanization process by APTES (figure 2a) and APDMES (figure 2b); in both cases, red shifts of spectra can be observed. The phenomenon is due to the formation of a thin silane film on pore walls, which increases the average refractive indexes of PSi layers. In particular, a red shift of 21 nm has been registered for microcavities treated by APTES and of 6 nm in the case of APDMES silanized structure. These different values

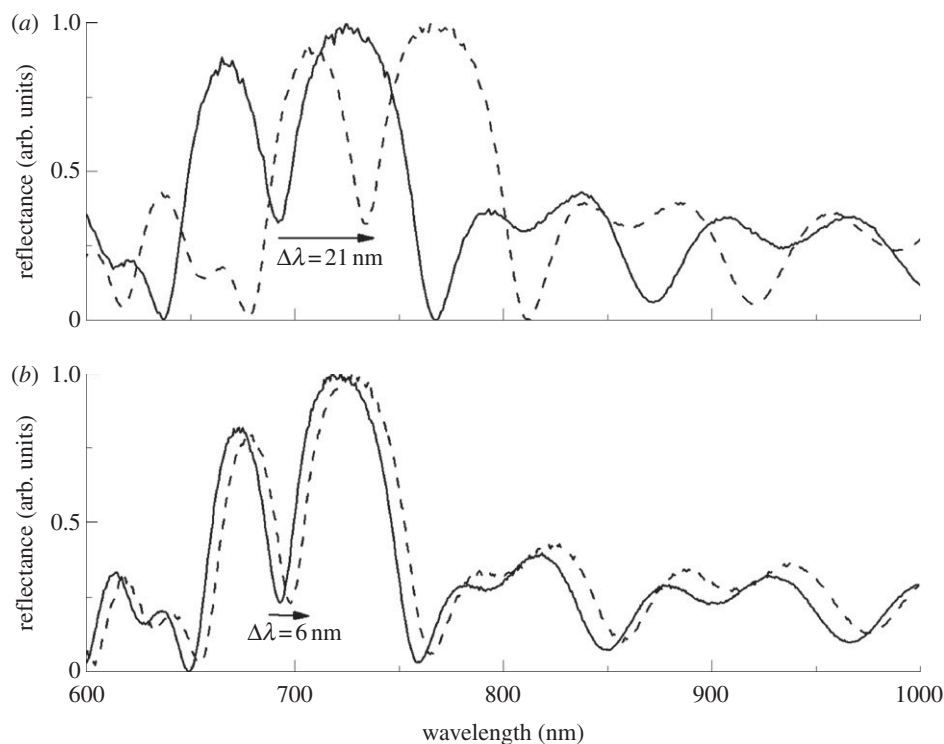


Figure 2. (a) Reflectivity spectra of a PSi microcavity before (solid line) and after (dashed line) the APTES functionalization. (b) Reflectivity spectra of a PSi microcavity before (solid line) and after (dashed line) the APDMES functionalization.

are due to diverse thicknesses (t) of silane layers coating internal surface of pores that we have quantified applying the following equation [19]:

$$\Delta n_{\text{pore}} = n_{\text{pore}}^{\text{after}} - n_{\text{pore}}^{\text{before}} \approx 4 \frac{t}{D} (n_{\text{layer}} - n_{\text{pore}}^{\text{before}}), \quad (3.1)$$

where Δn_{pore} is the increase in pore refractive index owing to the presence of silane layer, D the pore diameter, n_{layer} the refractive index of silane layer, $n_{\text{pore}}^{\text{before}}$ the refractive index of pores before silanization process. In our case, $n_{\text{pore}}^{\text{before}} = n_{\text{air}} = 1$, $n_{\text{layer}} = n_{\text{APTES}} = n_{\text{APDMES}} = 1.46(0.01)$, measured by spectroscopic ellipsometry (data reported in the electronic supplementary materials), and $D = 15$ nm. By changing thickness t (from 0.1 to 1 nm, by 0.1 step) in equation (3.1), we obtained a set of Δn_{pore} values, which have been used to calculate PSi microcavity spectrum red shifts [20]. Red shift observed in the case of APTES silanization (21 nm) corresponds to 0.7 nm thick layer computed ($\Delta n_{\text{pore}} = 0.086$); the lower shift measured for APDMES treatment (6 nm) corresponds to a layer with a calculated thickness of 0.2 nm ($\Delta n_{\text{pore}} = 0.024$). This result depends on the ability of APTES to polymerize, because it has three potential points to attach PSi substrate or other silane molecules, while APDMES cannot do it, as it has only one point to attach substrate (see binding schemes in figure 3) [21].

The control of surface wettability plays a key role in studies of biomolecule immobilization. We have carried out measurements of WCA on a bare oxidized PSi sample and on silanized samples. The oxidized PSi is strongly hydrophilic (figure 3a), resulting in a WCA value of $(12.6^\circ \pm 0.8^\circ)$; APTES, with its alkyl chain and three ethoxy groups, induces an increase in the WCA to $(34^\circ \pm 1^\circ)$ (figure 3b) corresponding to a reduction in the degree of hydrophilicity; APDMES, characterized by the same alkyl chain but two methyl groups, creates a surface with a higher WCA $(42^\circ \pm 4^\circ)$ (figure 3c).

After these preliminary characterizations, PSi- $L_{a,b}$ devices have been exposed to the same synthesis process based on phosphoramidite chemistry. It is possible to quantify functionalization yield of each synthesis cycle by ultraviolet (UV) spectroscopy after detritylation of 5'-dimethoxytrityl (DMT) group released in solution [8]. In figure 4a,b, we report functionalization yield for PSi- L_a and PSi- L_b samples, respectively: in both cases two regions, separated by a dashed line in each graph, can be distinguished.

In the first one, the yield is quite constant and high (approx. $100\% \pm$ experimental errors), whereas in the second one, there is a dramatic decrease: each solid phase synthesis step involves an increasing inefficiency as ON length is increased [22]. Efficiency of coupling to PSi- L_a (figure 4a) falls just after the fourth-base growth; this threshold is pushed after the seventh thymine in the case of PSi- L_b (figure 4b). In general, coupling yield to PSi- L_b is greater than that of PSi- L_a for all the synthesis cycles, up to 13 thymine, T13: this experimental result can be reasonably ascribed to higher steric hindrance of pores caused by thicker APTES film with respect to the APDMES one. UV intensity measurements at T13 (I_{T13} column of table 1) also enable the quantification of functionalization F in terms of mol g^{-1} for both samples (PSi- $L_{a,b}$ weight about 0.3 mg) by using the Lambert–Beer formula (molar absorptivity $\varepsilon = 71\,700 \text{ M}^{-1} \text{ cm}^{-1}$). Data obtained are reported in table 1 together with the specific surface area (SSA) of PSi- $L_{a,b}$. PSi samples ‘as etched’ are characterized by SSA values of about $100 \text{ m}^2 \text{ g}^{-1}$ measured by the Brunauer–Emmett–Teller (BET) method [8]; the KOH process induces an increase in porosity of about 6 per cent corresponding to an increase in SSA to $106 \text{ m}^2 \text{ g}^{-1}$, quantified by spectroscopic reflectivity (50 nm blue shift of the microcavity spectra, data not shown here). On the contrary, thermal oxidation decreases SSA by 60 per cent (from 106 to $43 \text{ m}^2 \text{ g}^{-1}$), owing the isotropic silica growth also into the pores [18]. PSi- $L_{a,b}$ SSA values (shown in

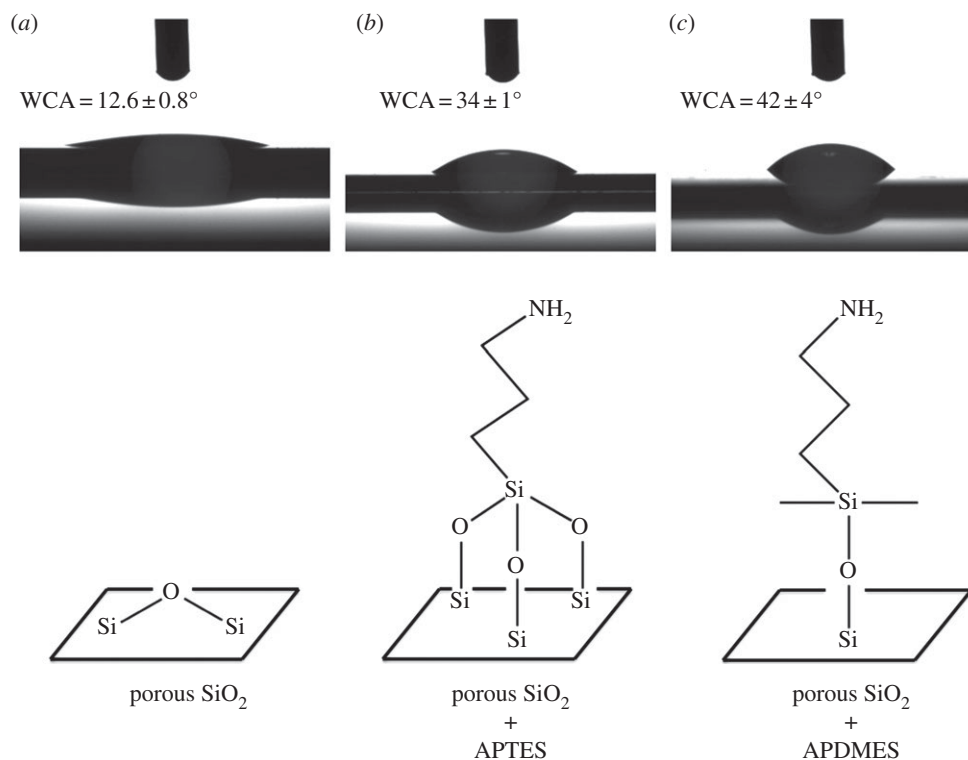


Figure 3. Water contact angle measurements performed on (a) oxidized PSi, (b) oxidized PSi modified with APTES and (c) oxidized PSi modified with APDMES.

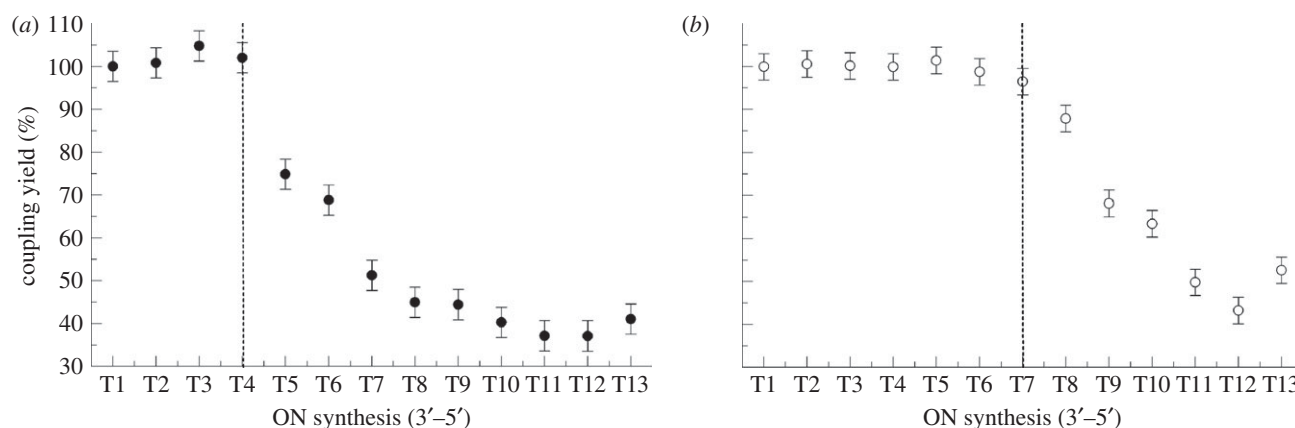


Figure 4. Coupling yield calculated by means of DMT measurements performed on the PSi samples silanized with (a) APTES (filled circles) and with (b) APDMES (open circles).

Table 1. Functionalization values calculated for the PSi structures modified with the two aminosilanes. I_{T13} , UV intensity recorded corresponding to the 13 coupling; F , functionalization in terms of mol g^{-1} or nmol cm^{-2} ; SSA, specific surface area.

aminosilane	I_{T13}	F (mol g^{-1})	SSA ($\text{m}^2 \text{g}^{-1}$)	F (nmol cm^{-2})
APTES	0.12 ± 0.01	$(2.6 \pm 0.2) \times 10^{-5}$	39	0.067 ± 0.005
APDMES	0.17 ± 0.01	$(3.7 \pm 0.2) \times 10^{-5}$	43	0.086 ± 0.005

table 1) have been calculated by applying a simplified model based on the cylindrical shape of the pores taking into account the thickness of silane layer on the pore walls (0.7 nm for APTES and 0.2 nm for APDMES). Sample functionalization in terms of nmol cm^{-2} , last column in table 1, is the ratio F/SSA .

The better coupling yield obtained for PSi-L_b results in higher number of moles per square centimetre with respect to the PSi-L_a sample (about 0.09 versus 0.07), but both

numbers are two order of magnitude less than those obtained in our previous work (3.2 versus 1.1) [8]. We explain this difference in terms of PSi support stability: hydrolysed oxidized PSi structures were slightly corroded during ON growth: at each cycle, new $-\text{OH}$ groups occupied the fresh surfaces of fractures, thus becoming binding sites for thymine bases. This is also the reason why we measured an almost constant yield for all the ON syntheses. In the present experiment, there is not any etching of the PSi surface during ON growth, so

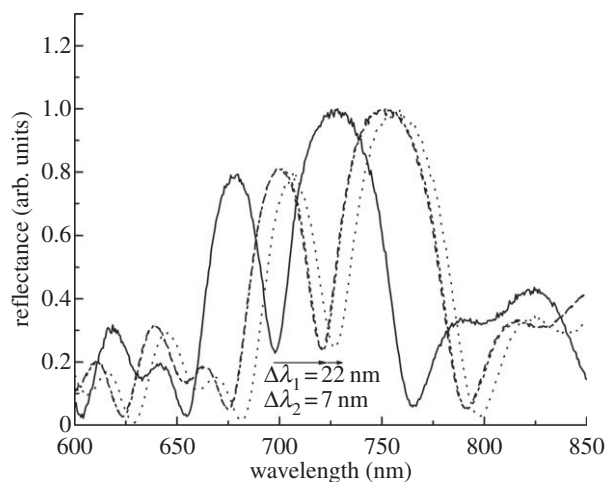


Figure 5. Reflectivity spectra of the PSi microcavity after the APDMES functionalization (solid line), after the T13 synthesis (dashed line), after the C20 incubation (short dashed line) and after the A20 incubation (dotted line).

that only linear chains of thymine bases are obtained: in other words, while 3.2 nmol cm^{-2} were constituted by T1–T13 sequences, mixed in unknown percentages, all $0.09 \text{ nmol cm}^{-2}$ are T13 ONs. From the reaction point of view, it is possible to obtain greater functionalization degree by pushing forward operational conditions, but, in the present study, our interest was to demonstrate the possibility of synthesizing ON sequences on a stable porous matrix, and monitoring the process by optical spectroscopy. Furthermore, chemical stability of the support material turns out to be a key issue for the hybridization experiment. In summary, PSi- L_b sample showed higher functionalization and less steric hindrance of pores, so that only this silanized multilayer has been selected as active substrate for the experiment of complementary sequence recognition, described in the following.

The hybridization to complementary (Fluo-dA20) and non-complementary (Fluo-dC20) ON sequences has been monitored by spectroscopic reflectometry, and, for comparison, also by fluorescence microscopy.

Figure 5 shows reflectivity spectra of device before synthesis process, after T13 synthesis and after complementary DNA interaction: a red shift of 22 nm has been detected after T13 synthesis, which is a value higher than 11 nm obtained in the case of oxidized multilayer [8]. This red shift increase is due to a higher chemical stability of the PSi platform during the synthesis process. The spectrum shifts a further 7 nm after the complementary DNA exposure, thus demonstrating the detection of DNA–DNA interaction. In figure 5, it is also possible to observe how exposure to non-complementary sequence does not induce any shift, confirming specificity of biosensor.

Optical monitoring of T13–A20 hybridization has also been done by fluorescence analysis.

In figure 6, we report bright field (figure 6*a*) and fluorescence (figure 6*b*) images of PSi- L_b -T13 surface after incubation with fluorescently labelled complementary (Fluo-dA20) and non-complementary (Fluo-dC20) sequences. While in bright field imaging surface samples appeared indistinguishable, switching from bright-light source to fluorescence-light source, we observed high fluorescence (maximum peak value = 73 counts; average intensity = 34 counts) only on exposure to complementary ON sequence; on the contrary, the chip incubated with non-complementary

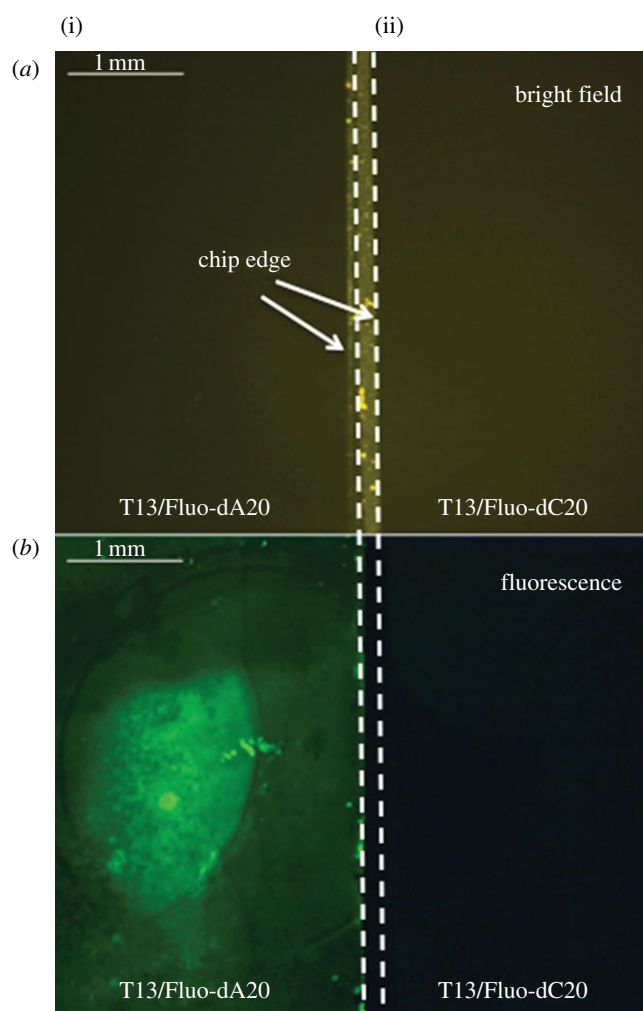


Figure 6. (a) Bright field and (b) fluorescence images of the PSi surface functionalized with T13 after the incubation with solutions (i) containing Fluo-dA20 and (ii) Fluo-dC20. Scale bar, 1 mm. (Online version in colour.)

sequence appeared completely dark (maximum peak value = 7 counts; average intensity = 5 counts).

4. Conclusions

Oxidized PSi multilayered structures are widely used as optical transducers in a lot of laboratories, and even commercial applications: biosensors are mostly exploited in real time monitoring of biomolecule interactions, and microarrays are great workhorses for genomic and proteomic studies. The main drawback of these cheap, easy-to-produce and sensitive devices is their low chemical stability in alkaline physiological solutions, actually used in biochemical experiments. In this work, we have investigated two common routes of passivation/functionalization by grafting alkylsilanes through the formation of Si–O–Si bonds between silanol groups induced on the oxidized PSi surface and hydrolysed organosilane molecules of APTES or APDMES. Both compounds, but with some differences, form a thin film on PSi surface, which can effectively protect the porous support against corrosion on exposure to weak basic environment ($\text{pH} = 7/8$). It is well known, in fact, that APTES can polymerize forming a multilayered interface, while APDMES cannot do this. Despite this not negligible distinction, we found that both silane-modified

PSi devices showed good chemical resistance to reagents used for *in situ* synthesis, phosphates-deprotection and hybridization of a polythymine ON. In particular, APDMES passivation, owing to less steric hindrance of the pores, results in a better functionalization quality with respect to APTES. Finally, hybridization to a target sequence of ON synthesized on APDMES modified porous silica optical structure has been demonstrated by complementary optical techniques, such as spectroscopic reflectivity and fluorescence microscopy. Since we have demonstrated the stability of aminosilanized PSi on exposure to carbonate/dry methanol solutions, the use of the so-called 'ultramild' phosphoramidites can be also

exploited in future experiments: this result will open the prospect of PSi solid phase ON synthesis without any restriction on the ON composition sequence, giving more general significance to the proposed methodology. In addition to biosensors and microarrays, solid-supported synthesis of ON conjugates will facilitate delivery and targeting of nucleic acid drugs, thus unlocking a very innovative and promising route to new kinds of chemotherapy and other exciting medical challenges [23].

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