ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 2116–2118

www.rsc.org/chemcomm

COMMUNICATION

DNA microarrays on silicon surfaces through *thiol-ene* chemistry†

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Received 23rd November 2011, Accepted 12th December 2011
DOI: 10.1039/c2cc17321b

The potential of *thiol-ene* chemistry as a selective strategy to functionalize silicon materials for DNA microarraying is demonstrated and applied to discriminate genetic variations.

Engineering of inorganic material surfaces with organic molecules has found widespread applications in materials and life science.[1] Chip-based analytical platforms, notably in a microarray format, are generally fabricated on glass, silicon, polymers or noble metal surfaces.[2] In this context, DNA immobilization onto a solid surface is a critical step to implement a variety of important technologies such as gene expression, biomolecular computation, advanced molecular devices, and biosensors.[3] Because of its attractive properties, silicon is commonly used for fabricating biochips.[4] Among the different organic compounds that can self-assemble on silicon surfaces, organosilanes are widely used to covalently attach promoters or cross-linkers.[5] When working with Si-based nanobiosensors, a selective attachment of the bioreceptor on the sensing area is of outmost importance to reach the optimal sensitivity.[6] In this context, the photoimmobilization is an effective and clean strategy to perform localized attachment of probes; as it has been recently demonstrated by Vong *et al.*[7] They locally patterned a silica microchannel by means of photochemical attachment of a trifluoroethyl 1-alkene ester by irradiation at 254 nm; and subsequently employed such a patterned channel to perform hybridization assays after immobilization of aminated DNA oligomers. The drawback of this methodology is that long irradiation times (10 h) are required. As an alternative, one attractive click reaction is the addition of thiols to alkenes, called *thiol-ene* coupling (TEC),[8] which takes place at close-to-visible wavelengths (λ = 365 nm) using short times (10 min). Very importantly, this procedure is compatible with aqueous media chemistry, which is crucial for its bioavailability. In the last few years, *thiol-ene* chemistry has also found important applications in the area of surface derivatization chemistry.[9]

In the present study, we focus on the preparation of biotinylated surfaces by means of thiol-ene chemistry. Next, streptavidin is used to immobilize biotinylated DNA probes. In this communication, target DNA hybridization and further SNP discrimination are demonstrated for the first time on a TEC silicon/silicon oxide functionalized surface.

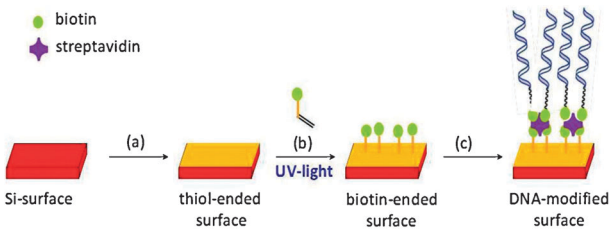

Fig. 1 Functionalization of Si/SiO_2 surface by *thiol-ene* chemistry. (a) 3-Mercaptopropyl trimethoxysilane 1% in toluene (2 h, rt); (b) biotin probes in DMSO (UV 365 nm, 10 min); (c) streptavidin and DNA probe (5 h).

For that, functionalized slides were prepared by following the scheme shown in Fig. 1. Olefin-functionalized derivatives **1–3** were used for photoimmobilization on thiol-functionalized surfaces, while compound **4** was used as control (Fig. 2).

Silicon oxide-coated silicon slides were cleaned with piranha solution and functionalized with 3-mercaptopropyl trimethoxysilane (1% in toluene). Next, biotin derivatives **1–4** were spotted onto thiol-functionalized slides and exposed to UV light (10 min) to induce the TEC. After washing, bovine serum albumin (BSA in PBS-T) was deposited to block against unspecific adsorption. The extent on the biotinylation was evaluated using ATTO-streptavidin and the fluorescence measured with a homemade luminescence surface reader.[10]

When the photoimmobilization efficiency of compounds **1–4** was compared (Fig. 3), it was observed that compound **1** gave a low fluorescence intensity. This might be attributed to the short distance between the biotin heterobicycle and the surface, which might hinder streptavidin binding. On the other hand, compounds **2** and **3**, bearing a tetraethylene-glycol-based olefinic

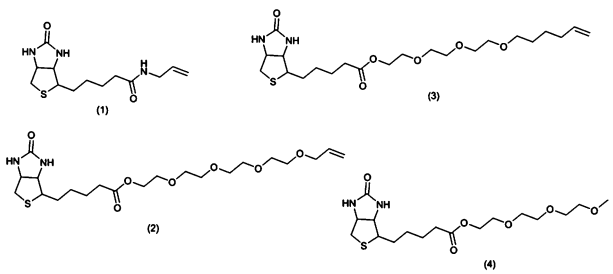

Fig. 2 Biotin derivatives employed in the TEC photoimmobilization.

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† Electronic supplementary information (ESI) available: Materials, instruments and experimental procedures. See DOI: 10.1039/c2cc17321b

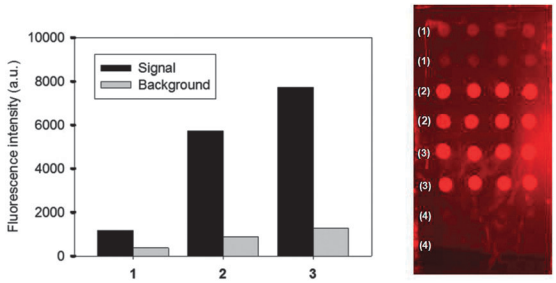

Fig. 3 Comparison of biotin derivatives **1**, **2** and **3** deposited on thiol-functionalized slides followed by photoimmobilization.

linker, gave comparably higher fluorescence intensities with an S/N ratio of 6. Finally, compound **4**, consisting of a biotin that lacked the olefin linker, showed negligible immobilization, discarding the nonspecific adsorption of biotin. A control assay dropped out the possibility of nonspecific adsorption of ATTO-streptavidin onto a thiol-functionalized slide under the used conditions.

Afterwards, different concentrations of compound **2** were studied and, as expected, fluorescence intensity increased with concentration (ESI†, Fig. S1). For 1 mM concentration, fluorescence intensity decreased and dilutions below 0.5 mM resulted in non-homogeneous spots, whereas solutions over 10 mM resulted in the signal saturation. A maximal S/N ratio of 5 was observed at 5 mM concentration. The pH required for streptavidin immobilization was also investigated, resulting the optimal at pH 7.5, although no significant differences were observed between pH 6 and 10 (ESI†, Fig. S2).

Microarray quantification was carried out by developing a standard calibration curve plotted between fluorescence intensity and the amount of spotted labeled protein (ESI†, Fig. S3). Under the studied conditions, immobilization yields up to 86.7% and protein surface coverage of 21.8 pmol cm^{-2} were reached.

In order to demonstrate the selectivity of the TEC strategy, compound **2** was spread out onto the thiol-functionalized slides, and immediately covered with a photomask. Subsequent irradiation led to patterning with adducts of covalently attached derivative **2**. As shown in Fig. 4, selective localized immobilization of **2** and further streptavidin recognition were achieved. These results show the advantage of this approach for the selective attachment of biomolecules on the surface, offering an alternative to the one reported by Vong *et al.*[7]

The functionalized surfaces were characterized using water contact angle (WCA), XPS and ATR-FTIR techniques. WCA increased to 65.5° after reaction with 3-mercaptopropyl trimethoxysilane. After photoimmobilization of compound **2**, the WCA slightly decreased to 59.9°, which might be attributed to the presence of ether groups from this derivative. Upon addition of streptavidin, the WCA decreased to 54.2°, as observed for similar systems.[11] In the XPS analysis (ESI†, Fig. S4) of the thiol-functionalized surface, the C1s signal can be deconvoluted into three components at 287, 286 and 285 eV, assigned to C–O, C–S and C–C carbon atoms, respectively (ESI†, Fig. S4B). It is noteworthy that the signal at 287 eV is attributed to the presence of methoxy groups from the silane. Bands corresponding to the characteristic stretching vibrations of CH_2 (~2913 cm^{-1}) and S–H (2571 cm^{-1}) were observed in the ATR-IR spectra. For the biotinylated surface, the C1s peak deconvolutes into an additional band at 289 eV corresponding to the C=O carbon atom (ESI†, Fig. S4B). All electron binding energies of the different types of carbon peak positions were derived from the literature for similar systems.[12] Finally, ATR-IR spectra showed bands at 1540 and 1681 cm^{-1}, confirming the presence of biotin moiety.

Afterwards, the optimal conditions for oligonucleotide immobilization were investigated. For that, solutions containing streptavidin and 3′-Cy5, 5′-biotin oligonucleotide (probe F, Table 1) at different molar ratios, namely 1 : 1, 1 : 2 and 1 : 3 were prepared in CB buffer. Microarrays were spotted over a biotinylated surface and subsequently incubated in a moist, dark chamber at room temperature. After washing, the maximum fluorescence intensity was obtained for the 1 : 2 streptavidin : probe F molar ratio (ESI†, Fig. S5). This may suggest that streptavidin is being attached to the biotinylated surface through two of its four active sites. In this way, the 1 : 2 ratio means the optimal relationship between an efficient streptavidin attachment and the maximum probe loading. Additionally, several incubation times (1, 5, and 15 h) were assayed. Significant fluorescence intensities and good S/N ratios (up to 7) were obtained for incubation times longer than 5 h. No significant fluorescence signal was obtained for arrays lacking streptavidin, confirming no unspecific adsorption of probe F.

It is well known that pH is a critical factor in the streptavidin–biotin recognition process. For that, an assay was carried out by employing solutions containing streptavidin and probe F (in a 1 : 2 molar ratio) in different buffers: SSC 1× (pH 7.4), CB 1× (pH 9.6) and PBS 1× (pH 11). Best results were obtained when using CB or PBS as printing buffers, whereas a significant decrease of the fluorescence intensity was observed when SSC buffer was used (ESI†, Fig. S6).

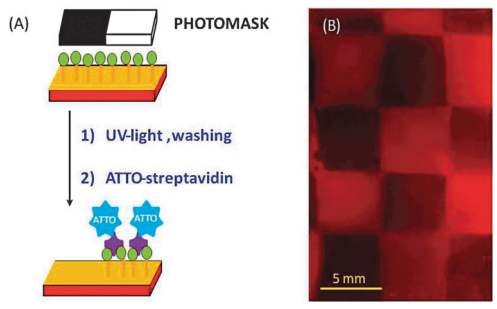

Fig. 4 (A) Schematic representation of photomasking. (B) Fluorescence image obtained.

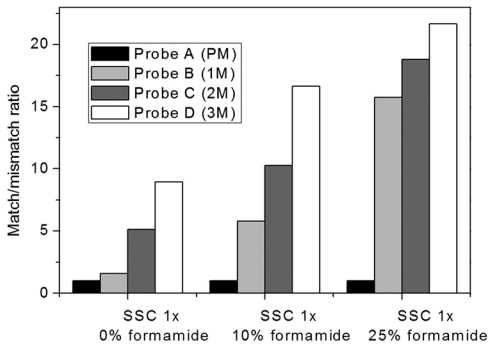

Fig. 5 Effect of formamide in the detection of SNPs.

Table 1 Nucleotide sequence of probes and targets used

Name	Sequence (5′ to 3′)	5′ end	3′end
Probe A	$(T)_{15}$-CCCGATTGACCAGCTAGCATT	Biotin	None
Probe B	$(T)_{15}$-CCCGATTGACCTGCTAGCATT	Biotin	None
Probe C	$(T)_{15}$-CCCGATTGATTAGCTAGCATT	Biotin	None
Probe D	$(T)_{15}$-CCATATTGACCAGCTATCATT	Biotin	None
Probe E	$(T)_{15}$-ATTGCTAGCTAATCAATCGGG	Biotin	None
Probe F	$(T)_{15}$-CCCGATTGATTAGCTAGCATT	Biotin	Cy5
Target A	AATGCTAGCTGGTCAATCGGG	Cy5	None

In order to quantify the density of immobilized oligonucleotide, solutions containing streptavidin and probe F in a 1 : 2 molar ratio were spotted onto a biotin-functionalized slide (concentrations ranging from 0.1 to 5.35 μM in CB 1×). After incubation and washing, probe F showed concentration-dependent immobilization, reaching densities up to 2 pmol cm^{-2} in the studied conditions (ESI†, Fig. S8). These results were in the range of those reported by other authors on different surfaces (ESI†, Table S2).[13]

The scope of the proposed methodology to perform hybridization assays was studied. First, mixtures containing streptavidin and 5′ biotin-oligonucleotide (probe A, Table 1) in 1 : 1, 1 : 2 and 1 : 3 molar ratios were dropped onto the slides. After incubation, washing and blocking with BSA, complementary oligonucleotide 5′ Cy5-labeled (Target A, Table 1) was dispensed (in SSC 1×) and incubated for 45 min in a moist chamber at 37 °C. After rinsing and drying, the fluorescence intensity was registered. Again, best results were obtained when using a 1 : 2 streptavidin : probe ratio (ESI†, Fig. S9). Control experiments without probe A were performed to discard unspecific adsorption of DNA target strands on the surface. Using the described procedure, it was possible to detect 10 pM target A concentrations.

The amount of hybridized DNA was obtained from the calibration curve (0.9 pmol cm^{-2}; ESI†, Fig. S10) and corresponds to 5.5×10^{11} molecules of DNA per cm^2, which is similar to the previously reported values on silicon,[14] gold,[15] and other substrates (ESI†, Table S2).[16] Depending on the immobilized probe density, the hybridization signal increased with the target concentration, reaching saturation between 200–500 nM of DNA. The maximum DNA target densities were estimated between 0.52 and 0.93 pmol cm^{-2} (ESI†, Fig. S11), with hybridization efficiencies ranging from 46% to 93%.

Finally, the ability to discriminate single nucleotide polymorphism (SNP), with potential application in predicting disease predispositions or drug responses, was evaluated.[17] In order to demonstrate the practical application of our platform for diagnostic purposes, the probe sequences designed for this study correspond to different serotypes of *Plum pox virus*, which is the most devastating viral disease of stone fruit from the genus *Prunus*. For that, hybridization was performed using a full complementary probe (probe A), three mismatched probes (probes B, C and D) and a noncomplementary probe (probe E) to a target Cy5-labeled oligomer (target A) at 0.5 nM concentration in SSC buffer. No measurable hybridization signal from the noncomplementary probe was detected. Working under the described hybridization conditions, the sensor was not able to discriminate between the perfect match target and the single base-pair mismatch target. But, a decrease of the ionic strength (0.1×) allowed us to discriminate even one single nucleotide mismatch (ESI†, Fig. S12). On the other hand, the fluorescence decreased for the mismatched probes as the formamide concentration increased, reaching maximum discrimination ratios (between 15.2 and 22.1) when 25% formamide was used (Fig. 5).

In conclusion, this study shows a patterned silicon surface modification for the attachment of biomolecules, by employing organosilane chemistry and TEC. This approach showed selective binding to streptavidin, offering a general method for the localized biofunctionalization. The method retains the physical properties of the support, and its application is versatile and clean. Working on a microarray format, immobilization densities of 2 pmol cm^{-2} and hybridization efficiencies higher than 90% were obtained. The excellent properties shown by this methodology enable us to discriminate SNPs for *Plum pox virus*, demonstrating its suitability for bioanalysis.

This work was supported by the Spanish Ministerio de Ciencia e Innovación (FEDER-CTQ2010-15943) and Generalitat Valenciana (PROMETEO/2010/008).

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