Escorihuela Fuentes, J.; Bañuls Polo, MJ.; Puchades Pla, R.; Maquieira Catala, Á. (2012). Development of oligonucleotide microarrays onto si-based surfaces via thioether linkage mediated by UV irradiation. Bioconjugate Chemistry. 23(10):2121-2128. doi:10.1021/bc300333a.

Development of Oligonucleotide Microarrays onto Si-based Surfaces via Thioether Linkage Mediated by UV Irradiation

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ABSTRACT

Selective covalent immobilization of thiolated oligonucleotides onto an epoxy-functionalized silicon-substrate can be achieved via light radiation (365 nm). Following this approach, thiol-modified oligonucleotide probes were covalently attached as microarrays, reaching an immobilization density of 2.5 pmol·cm^{-2}, with a yield of 53%. The developed method presents the advantages of, spatially controlled probe anchoring (by means of using a photomask), direct attachment without using cross-linkers and short irradiation times (10 min). Hybridization efficiencies up to 65%, with full complementary strands, were reached. The approach was evaluated by scoring single nucleotide polymorphisms with a discrimination ratio around 15. Moreover, sensitive and selective detection of bacterial *Escherichia coli* was demonstrated.

1. INTRODUCTION

The development of highly performance methods for the sensitive and selective detection of DNA and RNA targets has become a key point in biomedical studies (*1*). Especial emphasis has been focused on DNA microarrays as they allow continuous, fast, sensitive, and selective detection of DNA hybridization (*2*). Thus, DNA microarrays have many applications such as clinical diagnostics (*3*), gene expression (*4*), disease prevention (*5,6*), reaction discovery (*7*), forensic determination (*8*), and bioterrorism (*9*). DNA chip technology uses microscopic arrays of DNA molecules immobilized on solid supports, frequently glass (*10*), but the silicon-based materials are also used as biosensing platforms due to its favorable properties, such as biocompatibility, versatile chemical functionalization, huge surface-to-volume ratios, and mass production using the well established semiconductor microelectronic technology (*11*). Additionally, the use of silicon as solid support for the construction of DNA microarrays has several advantages among all the commonly employed surfaces. First, silicon wafers have less surface roughness, which allows better uniformity of DNA deposition with a higher density and smaller size of DNA spots. Second, silicon surfaces provide a better S/N ratio because silicon wafers show less background fluorescence. Finally, silicon chip-based DNA microarrays facilitate the fabrication of "lab-on-a-chip" devices (*12*).

One of the critical issues in the development of a biosensor is the effective immobilization of the DNA probe onto the solid support. The most commonly used methods for immobilizing a DNA or oligonucleotide probe to solid surfaces are adsorption, affinity interaction and covalent attachment. Among them, the last one has been established as the preferred approach on silicon because of the higher stability, reproducibility, probe directionality, reduces background noise, controlled immobilization and hybridization efficiency when compared to the noncovalent immobilization strategies (*13*). The modification of silicon-based materials for the fabrication of an effective biorecognition surface is typically accomplished via silane chemistry on the previously oxidized silicon surface. Although there are different organic molecules that can self-assemble on silicon surfaces and modify their intrinsic properties, silane compounds have been widely used to covalently attach adhesion promoters and cross-linkers onto these surfaces (*14*). In this regard, organosilane chemistry is applied to introduce amine, carboxyl, vinyl, or thiol groups onto the surface, which are used for further functionalization and for the immobilization of polymers or biomolecules (*15*). Among functionalized supports, epoxy-based surfaces are significantly interesting, as epoxides are known to be reactive toward electrophilic as well as nucleophilic groups. As example, Preininger et *al*. immobilized amine- and thiol-modified oligonucleotide probes onto a glass

slide coated with an epoxy resin; in this way they generated high-capacity surfaces used to detect PCR products from *Rhizobium freezi*. However, low reproducibility and high background signal were observed (*16*).

Thus, 3-glycidoxypropyltrimethoxy silane (GOPTS) has been widely used in the field of DNA microarrays in order to attach oligonucleotide probes covalently to the glass and silicon-based surfaces. The attachment of thiolated oligonucleotides on epoxylated silicon (111) surface by classical substitution reactions with immobilization efficiencies around 45% has been reported (*17*), but long incubation times, around 12 h, were required. Also, Mahajan *et al.*(*18*) proposed the covalent attachment of thiol-modified probes to epoxy-activated glass slides under microwave activation for 12 min reaching immobilization efficiencies up to 24%. Patnaik *et al.* (*19*) immobilized carboxyalkyl-modified oligonucleotides using the same epoxy-functionalized surfaces to develop biochips for diseases diagnostic.

The ability to pattern surfaces is a central topic of numerous chemical studies (*20*). In this regard, the use of selective irradiation through a photomask constitutes an easy approach to get spatially resolved chemical functionalities. The photoimmobilization of biomolecules onto surfaces has become a fast, smart and selective strategy to perform biomedical analysis and click reactions have also been successfully applied to functionalize different substrates (*21*). Light mediated reactions are fast clean and allow localized and selective attachment on the surface, which is critical when developing efficient biosensor. In our previous studies, we have applied *thiol-ene* chemistry as a selective strategy for functionalizing silicon-based platforms to develop DNA microarrays, showing excellent performances and indicating that light-induced reactions onto thiol groups result highly promising (*22*).

FIGURE 1

In this article, we describe the photoinduced covalent attachment of thiol-modified probes to epoxy-activated silicon-based surface. This strategy is a novel, clean and fast methodology to develop microarraying platforms applicable to nucleic acid detection. Under the described conditions, oligonucleotides have been successfully attached in a spatially controlled manner through a photomask irradiation. Furthermore, the constructed microarrays efficiently discriminated base mismatch and have been further used for the detection of bacterial *Escherichia coli* DNA.

EXPERIMENTAL PROCEDURES

Chemicals

The silicon-based wafers were provided by the Valencia Nanophotonics Technology Center (NTC) at the Universitat Politècnica de València (Spain) as 3-μm-thick silicon oxide layer grown on (100) silicon wafer. Hydrogen peroxide (35% w/w), 3-glycidoxypropyl dimethoxymethyl silane, 3-glycidoxypropyldimethylethoxy silane and 2-mercaptoethanol were purchased from Sigma-Aldrich Química (Madrid, Spain). 3-Glycidoxypropiltrimetoxy silane was from Acros (Barcelona, Spain). Toluene, dichloromethane and sulfuric acid 95-98% were purchased from Scharlau (Madrid, Spain). In all cases GOPTS was distilled previous to use and diluted in different solvents. Oligonucleotides sequences acquired from Thermo Fischer (Madrid, Spain) are shown in Table 1. *Escherichia coli* PCR products were kindly provided by Mobidiag (Helsinki, Finland). Note: All the chemicals should be handled following the corresponding material safety data sheets. The buffers employed phosphate buffer saline (PBS, 0.008 M sodium phosphate dibasic, 0.002 M sodium phosphate monobasic, 0.137 M sodium chloride, 2.7 M potassium chloride, pH 7.5), PBS-T (10×PBS containing 0.05% Tween 20), saline sodium citrate (10×SSC, 0.9 M sodium chloride, 0.09 M sodium citrate, pH 7) and carbonate buffer (10×CB, 0.5 M sodium carbonate, pH 9.6) and washing solutions were filtered through a 0.22 μm pore size nitrocellulose membrane from Whatman GmbH (Dassel, Germany) before use.

Table 1. Nucleotide Sequence of Probes and Targets Used.

Name	sequence (5' to 3')	5' end	3'end
Probe A	$(T)_{15}$-CCCGATTGACCAGCTAGCATT	SH	Cy5
Probe B	$(T)_{15}$-CCCGATTGACCAGCTAGCATT	SH	none
Probe C	$(T)_{15}$-CCCGATTGACC**T**GCTAGCATT	SH	none
Probe D	$(T)_{15}$-CCCGATTGAC**TT**GCTAGCATT	SH	none
Probe E	$(T)_{15}$-CC**AT**ATTGACCAGCTA**T**CATT	SH	none
Probe F	$(T)_{15}$-CCCGATTGACCAGCTAGCATT	NH_2	Cy5
Probe G	$(T)_{15}$-CGCCGATAACTCTGTCTCTGTA	SH	none
Probe H	$(T)_{15}$-TTCACGCCGATAACTCTGTCTCT	SH	none
Target A	AATGCTAGCTGGTCAATCGGG	Cy5	none

Instruments

Microarray printing was carried out with a low volume non-contact dispensing system from Biodot (Irvine, CA, USA), model AD1500. Contact angle system OCA20 equipped with SCA20 software was from Dataphysics Instruments GmbH (Filderstadt, Germany). The measurements were done in quintuplicate at room temperature with a volume drop of 10 μL employing 18mΩ water quality. X-ray photoelectron spectra were recorded with a Sage 150 spectrophotometer from SPECS Surface Nano Analysis GmbH (Berlin, Germany). Non-monochromatic Al Kα radiation (1486.6 eV) was used as the X-ray source operating at 30 eV constant pass energy for elemental specific energy binding analysis. Vacuum in the spectrometer chamber was 9×10^{-9} hPa and the sample area analyzed was 1 mm^2. Atomic force microscopy (AFM) images were obtained with a Veeco model Dimension 3100 Nanoman from Veeco Metrology, (Santa Barbara, CA) using tapping mode at 300 kHh. Imagining was performed in AC mode in air using OMCL-AC240 silicon cantilevers (Olympus Corporation, Japan). The images were captured using tips from Nano World with a radius of 8 nm. Automated Mask Alignment System from EVG model EVG620 was employed to irradiate UV light. The fluorescence signal of the spots was registered with a homemade surface fluorescence reader (SFR) having a high sensitive charge couple device camera Retiga EXi from Qimaging Inc, (Burnaby, Canada) with light emitting diodes Toshiba TLOH157P as light source (*23*). For microarray image analysis and subsequent quantification, GenePix Pro 4.0 software from Molecular Devices, Inc. (Sunnyvale, CA, USA) was employed.

Silanization of slides

Si-based wafers were cut into pieces of 2×1 cm^2 and cleaned with piranha solution (H_2SO_4:H_2O_2 3:1 v/v) for 1 h at 50 ºC to remove organic contaminants. Caution: Piranha solutions react violently with organic materials and should be handled with extreme care. Then the chip was washed with deionized water and air dried. For achieving the surface functionalization, the chip was immersed under an argon atmosphere into a solution of 3-glycidoxypropiltrimetoxy silane 2% in toluene for 2 h at room temperature. Then the chip was washed with CH_2Cl_2 and air dried. Finally, the chips were baked for 10 min at 150 ºC and stored under inert atmosphere.

Oligonucleotide immobilization

Silicon-oxide slides were treated following the above-described procedure to obtain the corresponding epoxy-functionalized slides. To perform this study, oligonucleotide probe A (Table 1), consisting in 5' SH-, 3' Cy5 oligomer of sequence $(T)_{15}$-(CCC GAT TGA CCA

GCT AGC ATT) was used to evaluate the efficiency of the platform towards oligonucleotide immobilization. For that, different probe A solutions were prepared (40 nL) onto the epoxy-functionalized surface and exposed to UV-light at 365 nm (6 mW/cm^2) for 10 min through a photolithography apparatus to induce the immobilization. Finally, slides were thoroughly rinsed with PBS-T and water and air dried. Immobilization results were obtained from the fluorescence signals using the SFR.

Hybridization assays

To study the hybridization efficiency on the developed platform, silicon-based slides were functionalized with GOPTS as described above. Afterwards, solutions containing oligonucleotide 5' SH-labeled (probe B) were spotted (40 nL) onto the functionalized slides creating the microarrays. Then slides were exposed to UV-light at 365 nm (6 mW/cm^2) for 10 min. The slides were washed with PBS-T (15 min) and water (5 min) and air-dried. At this point, 50 μL of 2-mercaptoethanol 0.1M in CB 1× was spread under a coverslip and irradiated with UV light to block the remaining active sites. After washing, 50 μL of the complementary oligonucleotide 5' Cy5-labeled (target A) dissolved in SSC 1× were spread under a coverslip and incubated in a dark and humidified chamber for 1 h at 37 ºC. After rinsing and drying, the fluorescence intensity of the spots was registered with the SFR. Measurements were made by accumulation of emitted light by the samples during 15 seconds with a device gain of 5.

Oligonucleotide surface density

The immobilization density was quantified using fluorescence detection with probe A. Hybridization density was determined through fluorescence detection of target A after hybridization with probe B. For that, calibration curves were made by detecting the fluorescence of 50 nL dilutions dispensed on activated surfaces at concentrations ranging from 1 to 100 nM. The amount of immobilized and hybridized DNA was obtained from interpolation in the respective calibration curves. The yield of DNA hybridization was calculated as the ratio of the target to probe densities.

Mismatches detection

To investigate the sensitivity of the system, four oligonucleotides sequences, namely, SH-$(T)_{15}$-(CCC GAT TGA CCA GCT AGC ATT) (probe B), SH-$(T)_{15}$-(CCC GAT TGA CCT GCT AGC ATT) (probe C), SH-$(T)_{15}$-(CCC GAT TGA TTA GCT AGC ATT) (probe D) and SH-$(T)_{15}$- CCA TAT TGA CCA GCT ATC ATT) (probe E) having zero, one, two and three base mismatches, respectively, were spotted (40 nL/spot, 0.5μM) with a noncontact dispenser onto an epoxy-functionalized silicon oxide chip creating matrix of 4×5 spots. After

immobilization and blocking as described above, the microarray was subjected to hybridization with a labeled oligomer (target A), Cy5-(AAT GCT AGC TGG TCA ATC GGG) in SSC under different conditions for 1 h at 37 ºC. After washing and drying, the fluorescence was detected by SFR.

Detection of bacterial *Escherichia coli*

Silicon-based slides were functionalized with GOPTS as described above. Then, solutions containing SH-labeled probe G (*E. coli* specific probe) and probe H (control probe) were spotted onto the functionalized slides creating the microarrays of 4×4 spots, alternating rows of probe G (odd rows) with probe H (even rows) . Afterwards, slides were exposed to UV-light at 365 nm for 10 min and subsequently washed and air-dried. After blocking the remaining active sites with 2-mercaptoethanol 0.1 M in PBS 1×, Cy5-labeled PCR product solutions (50 μL) in hybridization buffer (SSC 1×) were distributed on the chip. PCR duplexes were firstly melted by 5 min incubation at 95 °C followed by fast cooling for 1 min on ice. After incubating 1 h at 37 °C, the slides were washed with PBS-T, rinsed with deionized water, and air dried.

3. RESULTS AND DISCUSSION

Immobilization of thiolated oligonucleotides onto epoxy-functionalized Si-based surfaces

Initially, a study of the silanization process was carried out after a surface oxidation pre-treatment using piranha solution. Organosilane reagents are commonly used to functionalize silicon oxide. Thus, the silanization time, using GOPTS 2% in toluene under argon atmosphere was optimized. Initially, a rapid increase of the water contact angle (WCA) was observed during the first 10 min, followed by a slight variation for the first 2 h, reaching a plateau at 4 h. For that, the final conditions were set at 2 h. (see Supporting Information Figure S1).

Then, the application of these epoxy-functionalized Si-based surfaces for selective covalent immobilization of thiolated oligonucleotide probes, using UV activation and further development of DNA hybridization assays, was demonstrated for the first time. As shown in Figure 1, a surface consisting of 3-μm-thick silicon oxide layer grown on (100) silicon wafer was first functionalized with GOPTS and, afterwards the 5’ SH-modified oligonucleotide probe was covalently attached through a thioether linkage. The immobilization under both UV and thermal conditions was compared by spotting probe A (Table 1) onto the epoxy-functionalized surface. The results showed that the reaction between the thiolated probe and epoxy groups is reached in 10 min under UV radiation, while 10 h at 37 °C were needed

under thermal conditions (Figure 2). It is noticeable, that in this short period of time no fluorescence was detected in the absence of UV light. It has been reported, that the kinetics of this reaction can be accelerated by means of microwave radiation (*18*), which can generate radicals, as well as UV light, that may react with the epoxy group and complete the reaction in several minutes. Additionally, to further investigate the oligonucleotide immobilization and optimize the optimal conditions, several irradiation times were assayed, and no significant differences in fluorescence were obtained for times longer than 10 min. Therefore, this irradiation time was applied for the rest of experiments.

FIGURE 2

The optimal buffer and pH conditions required for effective immobilization of the labeled oligonucleotide (probe A) were investigated because the reactivity of the epoxy group to a number of nucleophiles for immobilization can be influenced by the pH (*24*). Consequently, the photoinduced attachment was studied under different pH values (from 6 to 12). The results showed slight influence of pH on the immobilization of probe A, and maximum immobilization was achieved using PBS 1×, pH 6 (see Supporting Information Figure S2). As the pH was increased from 6 to 12, the fluorescence intensity decreased, which might be attributed to partial oxidation of thiol functions at higher pH values. In the case of the reaction mediated by thermal activation, maximum fluorescence was obtained for pH values around 9. It has been reported that alkaline conditions are needed to reach good density of the non protonated thiol group of the modified oligonucleotide and for a nucleophilic attack to occur at the functionalized surface (pKa of aliphatic thiol is approximately 8.0) (*25*).

The hydrophilicity of the treated surfaces causes the spreading of the printed spots increasing the evaporation speed, hindering reaction of probes with the surface. To control that issue, 5% glycerol was added to the printing solution.

Usually, the chemistry of the reactive organosilicon compounds R_nSiX_3-n, (X methoxy or ethoxy) is complex and it has been shown that the condensation reaction at a surface depends sensitively on the structure of the silane compound (*26*). The influence of the silane on DNA immobilization was also investigated by using several 3-glycidoxypropylalkoxysilanes having zero, one and two inactive alkyl groups (Figure 3A). For that, 3-glycidoxypropyltrimethoxy silane (**1**), 3-glycidoxypropyl dimethoxymethyl silane (**2**) and 3-glycidoxypropyldimethylethoxy silane (**3**) were used to functionalize the surface after piranha activation. Then, probe A was spotted onto the modified surface and irradiated with UV light

to photoinduce the attachment, and after washings, the fluorescence measured. The influence of the silane functionality was in agreement with previous studies with other silanes (*27*). This can be seen in Figure 3B, higher fluorescence intensities were obtained in the case of the trifunctional (**1**) and difunctional (**2**) silanes as compared to the monofunctional silane (**3**); reaching maximum intensities when silane **1** was used.

FIGURE 3

To investigate the oligonucleotide immobilization efficiency, probe A was serially diluted to several concentrations (ranging from 0.7 to 5 μM) and spotted onto the functionalized slide (see Supporting Information Figure S3). The immobilization efficiency was stablished from the standard calibration curve. Under the studied conditions (2.5 μM of spotted probe, see Supporting Information Figure S4), immobilization efficiencies up to 52.7% corresponding to immobilization densities around 2.5 $pmol \cdot cm^{-2}$ were reached for. This density was similar to that reported by others authors working on silicon-based materials (*28,29*).

The developed strategy based on UV light activation was compared with the epoxy-amine methodology (*30*). For that purpose, two immobilization experiments were run in parallel on two epoxy-modified chips. On the first one, probe A was spotted and immobilized following the optimized procedure. For the second one, a Cy5 labeled oligonucleotide, containing the same sequence than probe A but 3' NH_2-modified (probe F), was incubated for 8 h. After immobilization, slides were subjected to usual washing and drying. The immobilization and hybridization efficiencies of the proposed method (53%) were found to be significantly higher than the method employing epoxide-amine chemistry (12%), which shows the potential of the proposed methodology (see Supporting Information Figure S5).

The light induced immobilization is fast, clean and permits the patterning of the surfaces by using a photomask. This photopatterning is especially interesting when developing micro and nanobiosensors, where a selective attachment of the bioreceptors on the sensing area is of outmost importance to reach the optimal sensitivity. To demonstrate the patternig of surfaces, probe A at 3 μM in PBS was spread out onto the epoxy-functionalized slides, which were covered immediately with a photomask. After irradiation and washings, the slide was read with the SFR. As can be seen in Figure 4, selective immobilization of DNA probe can be achieved by means of UV irradiation in a simple, clean, fast and efficient way. It is noteworthy, that although previous strategies describing the covalent immobilization of thiolated oligonucleotides onto epoxy-functionalized surfaces have been described by

conventional nucleophile attack, to our knowledge this is the first time that light-induced selective immobilization is reported employing these reagents. On the other hand, the photoimmobilization is an effective and clean strategy to perform localized attachment of probes, as it has been recently demonstrated by Vong et *al.* (*31*) They locally patterned a silica microchannel by means of photochemical attachment of a trifluoroethyl 1-alkene ester by irradiation at 254 nm, and employed such patterned channel to perform 30 hybridization assays after immobilization of aminated DNA oligomers. Using such alkenylated surface and aminated oligonucleotides, long irradiation times (10 h) were required. In our case, only 10 min were needed to achieve efficient oligonuclotide attachment, featuring an important advantage regarding to this and other reported methods for DNA covalent immobilization on silicon-based materials.

FIGURE 4

Surface Characterization

The chemical surface functionalization was characterized by X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM) and water contact angle (WCA) measurements.

For all the raw material and treated surfaces the WCA was measured in ambient atmosphere at room temperature. The WCA of different samples was determined as the averaged of at least five repeat measurements for each sample. The hydroxylated silicon substrate before treatment with the piranha solution showed a WCA of about 33.5º. The hydrophilicity is attributed to the outer oxygen groups in the surface. After treatment with the piranha solution, it a WCA equal to 0, which is attributed to the high density surface hydroxyl groups generated with the piranha treatment. Upon reaction with GOPTS, WCA increased to 46.0º, in accordance with the presence of a more hydrophobic surface. After DNA immobilization, WCA increased to 51.1º, as observed for similar systems (50º for DNA immobilization on catecholamine polymer coated Si surfaces) (*32*).

The chemical composition change of the silicon oxide surfaces after functionalization was also investigated by XPS (see Supporting Information Figure S6 and Table S1). As expected, the Si 2p spectra for raw material and epoxy- and DNA-functionalized surfaces showed a sharp peak at 102-105 eV that is characteristic of oxidized silicon. For both functionalized surfaces, a superposition resulting from the different varying chemical environments of the

corresponding C atom was observed in the C 1s peak spectra (Figure 5). C1s signal for the epoxy-functionalized surface can be deconvoluted into three components at 288, 286 and 285 eV, that are assigned to C-O and C-C carbon atoms, respectively (Figure 5A). For the DNA-functionalized surface, the C1s spectrum was decomposed into four different carbon positions with varying intensities. Thus, to the bands of 287, 286 and 285 eV, assigned to C-O, C=O and C-C carbon atoms, additionally new band at 289 eV corresponding to C-N carbon atom is present (Figure 5B). This confirmed the presence of DNA. The XPS data also show the attachment of DNA on the surface with a strong peak in the N 1s region and an increase in the intensity of the C 1s peak at 288 eV characteristic of carbons associated with electron withdrawing groups such as nitrogen and oxygen (see Supporting Information, Figures S7 and S8). All electron binding energies of the different type of carbon peak positions were derived from literature for other similar systems(*33*).

FIGURE 5

Surface sample roughness was measured by AFM in order to obtain reliable data from the different functionalized surfaces. Figure 6 shows AFM images of typical clean SiO_2 surface after treatment with piranha solution (A) and a uniform monolayer after treatment with GOPTS (B) and DNA functionalization (C). Unmodified Si wafers were relatively flat, but distinctive circular and oval particles were visible as islands. These defects were similar to those observed by Wong et *al*.(*34*) and may have been etched back by the piranha treatment. The surface roughness changed after the GOPTS treatment and AFM images confirmed a complete surface coverage (see Supporting Information, Table S3). The roughness of bare surface was evident from its RMS surface roughness of 0.21 nm. In order to compare the surface roughness of the probe area to the silanized surface between the probes, the peak heights and RMS values of the surfaces were calculated. Difference between the peaks and the planes for the silanized silicon substrate was 1.34 nm with an RMS value of 0.37 nm, while the peak height after DNA probe attachment increased to 3.84 nm with an RMS value of 1.14 nm, indicating the effective coverage of oligonucleotide probe to the surface, which suggests that the probe is in a flattened configuration (*35*).

FIGURE 6

DNA Hybridization Assays

In order to test the hybridization capability of the developed surfaces, microarrays were prepared by immobilizing oligonucleotide probe B under the optimized conditions. Then, chips were blocked with 2-mercaptoethanol (0.1 M in CB) to deactivate the remaining active sites. After hybridization with target A for 60 min at 37 ºC and washing, fluorescence was read. No signal was obtained when the complementary oligonucleotide was incubated onto a functionalized surface lacking of probe B, demonstrating that nonspecific adsorption of target did not occur. The hybridization experiment performed at 37 ºC for 60 min showed significant hybridization, beyond which no appreciable increase occurred. Once established the time in 60 minutes, hybridization experiment was performed at various temperatures ranging from 30 to 50 ºC showing the best results from 40 to 45 ºC. The yield of hybridization was determined by comparison of the response obtained for target A and the response tor he immobilization of probe. As shown in Figure 7A, hybridized target density increased with target concentration, reaching a plateau at 200 nM for the tested probe concentrations and a maximum DNA target density of 1.4 pmol·cm^{-2} (probe, 5 μM; target, 200 nM) obtained from the interpolation in the corresponding calibration curve (see Supporting Information, Figure S10). The lowest detectable concentration for the target DNA recognized by the platform was 200 pM, which means a similar sensitivity than other reported surfaces (*36*).

In order to test the stability of the probe-coated surface, weekly hybridization assays were performed. The immobilized probes on GOPTS-functionalized silicon surfaces were active during, at least, six weeks after being printed on the chips without significant loss (15%) in the hybridization signal.

FIGURE 7

The selectivity of our approach was evaluated through hybridization with different oligonucleotides probes containing mismatched sequences towards target A. The ability to discriminate single nucleotide polymorphism (SNP) has important applications in predicting disease predispositions, drug responses in individuals (*37*) and for genetic analysis in drug discovery (*38*). In this assay, different oligonucleotide probes, consisting in a full complementary probe (B) and three mismatched probes (C, D and E) were immobilized onto a functionalized slide. All the probes contained a spacer tail of 15 thymines at the 5' end to physically separate the sequence from the surface, avoiding steric interferences (*39*). After washing and blocking with 2-mercaptoethanol, the hybridization with target A at 0.5 nM

concentration was done. Under the studied hybridization conditions (SSC 1×), it was not possible to discriminate satisfactory perfect match from single base mismatch target. But working under stringency conditions (*40*), by adding different formamide contents ranging from 0 to 30% (v/v), discrimination was possible. The addition of formamide at 30% (v/v), allows maximum discrimination ratio of 14.5 (Figure 7B). This result is in the range of those achieved with other approaches for oligonucleotides of similar length (*41*). Negligible responses (S/N < 3) were obtained assaying 5 and 10 base-pair mismatch targets for a broad range of concentrations (from 0.5 to 200 nM). An increase of the ionic strength of the hybridization buffer (3×) resulted in a worsening of the discrimination efficiency (see Supporting Information Figure S12), whereas a decrease of the ionic strength (0.1×) allowed us to discriminate even one single nucleotide mismatch lowering the formamide content (see Supporting Information Figure S13).

Finally, the response of the microarray platform for sensitive and selective detection of PCR amplified DNA products of pathogens was tested. The nucleotide sequence of the immobilized probe G was complementary to the central region of 300 bp amplicon specific to detect an innocuous serotype of *Escherichia coli*, a versatile bacterium with a number of unique features (*42*). Although most *E. coli* strains are harmless, some serotypes are pathogenic and can cause serious food poisoning in humans. Thus, the specific probe of the *E. coli* gene (probe G) and a noncomplementary probe (probe H), as control, were immobilized onto an epoxylated chip, and subjected to hybridization with the Cy5-labeled PCR amplicons. The PCR product concentration was determined by UV-Vis spectroscopy, resulting 46.21 ng/μL. A dilution of 1/1000 ratio was employed for the hybridization assays. Spots pertaining to probe sequence specific to *E. coli* gene of bacteria showed fluorescence (Figure 8), while lines containing the control spots pertaining to the control did not show fluorescence at all, showing specific detection of the system to prepare biochips for the detection of bacterial *E. coli.*

FIGURE 8

4. CONCLUSIONS

In summary, a simple and efficient method for preparation of oligonucleotide microarrays via light-activated thioether linkage is reported for the first time. Immobilization yields up to 53% under very short times (10 min) and hybridization efficiencies of 65% are reached. The

methodology shows potential for the construction of microarray platforms for DNA diagnosis, as demonstrated with the proof-of-concept detection for PCR products of *E. coli*. The main features of the proposed approach are the short times required (only 10 min) for oligonucleotide immobilization and the cleanness (no subproducts are generated). Also it permits the patterning in the biofunctionalization, which is highly interesting in the construction of high-Q nanobiosensors, where the selective attachment of the bioreceptors on the sensing area is a key step to yield optimal sensitivity performances. This strategy potentially simplifies the preparation of DNA microarrays and may be useful for the immobilization of different types of biomolecular probes, such as cDNA, peptides, aptamers, or direct polymerase chain reaction (PCR) products.

5. ACKNOWLEDGMENTS

Financial support from Ministerio de Ciencia e Innovación (CTQ2010-15943 BQU) and Generalitat Valenciana (programa PROMETEO/2010/008) is acknowledged. We also thank the NTC of the Universitat Politècnica de València for the clean room facilities.

REFERENCES

(1) Dolatabadi, J. E. N., Mashinchian, O., Ayoubi, B., Jamali, A. A., Mobed, A., Losic, D., Omidi, Y., and de la Guardia, M. (**2011**) Optical and electrochemical DNA nanobiosensors. *Trends Anal. Chem.* 30, 459-472.

(2) Sassolas, A., Leca-Bouvier, B. D. and Blum, L. J. (**2008**) DNA biosensors and microarrays. *Chem. Rev*. 108, 109-139.

(3) Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B. L., Mak, R. H., Ferrando, A. A., Downing, J. R., Jacks, T., Horvitz, H. R., and Golub, T. R. (**2005**) MicroRNA expression profiles classify human cancers. *Nature*, 435, 834-838.

(4) Bratu, D. P., Cha, B.-J., Mhlanga, M. M., Kramer, F. R., and Tyagi, S. (**2003**) Visualizing the distribution and transport of mRNAs in living cells. *Proc. Natl. Acad. Sci. U.S.A.*, 100, 13308-13313.

(5) Arora, K., Prabhakar, N., Chand, S., and Malhotra, B. D. (**2007**) Escherichia coli genosensor based on polyaniline. *Anal. Chem*., 79, 6152-6158.

(6) van't Veer, L. J., Dai, H. Y., van de Vijver, M. J., He, Y. D. D., Hart, A. A. M., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R., and Friend, S. H. (**2002**) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415, 530-536.

(7) Kanan, M. W., Rozenman, M. M., Sakurai, K., Snyder, T. M., and Liu, D. R. (**2004**) Reaction discovery enabled by DNA-templated synthesis and in vitro selection. *Nature*, 431, 545-549.

(8) Hopwood, A. J., Hurth, C.; Yang, J., Cai, Z., Moran, N., Lee-Edghill, J. G., Nordquist, A., Lenigk, R., Estes, M. D., Haley, J. P., McAlister, C. R., Chen, X., Brooks, C., Smith, S., Elliott, K., Koumi, P., Zenhausern, F., and Tully, G. (**2010**) Integrated Microfluidic System for Rapid Forensic DNA Analysis: Sample Collection to DNA Profile. *Anal. Chem.*, 82, 6991-6999.

(9) Rasooly, A., and Herold, K. E. (**2008**) Nano and Microsensors for Chemical and Biological Terrorism Surveillance, (Tok, J. B. H., Ed.), pp 116-165, Chapter 6, Royal Society of Chemistry, Cambridge.

(10) Lovrinovic, M. and Niemeyer, C. M. (**2005**) DNA microarrays as decoding tools in combinatorial chemistry and chemical biology. *Angew. Chem., Int. Ed*. 44, 3179-3183.

(11) Pasquarelli, A. (**2008**) Biochips: technologies and applications. *Mater. Sci. Eng. C* 28, 495-508.

(12) Lenigk, R., Carles, M., Ip, N. Y., and Sucher, N. J. (**2001**) Surface Characterization of a Silicon-Chip-Based DNA Microarray. Langmuir, 17, 2497-2501.

(13) Du, Q., Larsson, O., Swerdlow, H. and Liang, Z. (**2006**) DNA immobilization: silanized nucleic acids and nanoprinting. *Top. Curr. Chem*. 261, 45-61.

(14) Krupke, R., Malik, S., Weber, H. B., and Hampe, O. (**2002**) Patterning and visualizing self-assembled monolayers with low-energy electrons. *Nano Lett. 2*, 1161-1164.

(15) Plueddemann, E. P. (**1991**) Silane Coupling Agents. Springer: New York.

(16) Preininger, C., Sauer, U., Dayteg, J., and Pichler, R. (**2005**) Optimizing processing parameters for signal enhancement of oligonucleotide and protein arrays on ARChip epoxy. *Bioelectrochemistry* 67, 155–162.

(17) Böcking, T., Kilian, K. A., Gaus, K., and Gooding, J. J. (**2006**) Single-step DNA immobilization on antifouling self assembled monolayers covalently bound to silicon (111). *Langmuir* 22, 3494-3496.

(18) Mahajan, S., Sethi, D., Seth, S., Kumar, A., Kumar, P., and Gupta, K. C. (**2009**) Construction of Oligonucleotide Microarrays (Biochips) via Thioether Linkage for the Detection of Bacterial Meningitis. *Bioconjugate Chem*. 20, 1703-1710.

(19) Patnaik, S., Dash, S. K., Sethi, D., Kumar, A., Gupta, K. C. and Kumar, P., (**2012**) Engineered Polymer-Supported Synthesis of 3′-Carboxyalkyl-Modified Oligonucleotides and Their Applications in the Construction of Biochips for Diagnosis of the Diseases. *Bioconjugate Chem*. 23, 664-670.

(20) Rothemund, P. W. K. (**2006**) Folding DNA to create nanoscale shapes and patterns Nature, 440, 297-302.

(21) Hoyle, C. E., Loweb, A. B., and Bowman, C. N. (**2010**) Thiol-click chemistry: a multifaceted toolbox for small molecule and polymer synthesis. *Chem. Soc. Rev*. 39, 1355-1387.

(22) Escorihuela, J., Bañuls, M. J., Puchades, R., and Maquieira, A. (**2012**) DNA microarrays on silicon surfaces through *thiol-ene* chemistry. *Chem. Commun*., 48, 2116-2118.

(23) Mira, D., Llorente, R., Morais, S., Puchades, R., Maquieira, A., and Marti, J. (**2004**) High throughput screening of surface-enhanced fluorescence on industrial standard digital recording media. *Proc. SPIE*, 5617, 364-373.

(24) Hermanson, G. T. (**1996**) Bioconjugate Techniques; Academic Press, San Diego, CA.

(25) Tajc, S. G., Tolbert, B. S., Basavappa, R., and Miller, B. L. Direct Determination of Thiol pKa by Isothermal Titration Microcalorimetry (**2004**) J. Am. Chem. Soc., 126, 10508-10509.

(26) Del Campo, A., Bruce, I. J. (**2005**) Immobilisation of DNA on Chips I (Wittmann, C., Ed) Vol. 260, pp 86-88, Springer-Verlag Berlin Heidelberg.

(27) Solomun, T., Mix, R., and Sturm, H. (**2010**) Immobilization of Silanized DNA on Glass: Influence of the Silane Tether on the DNA Hybrydization. *ACS Appl. Mater. Interfaces* 2, 2171-2174.

(28) Chrisey, L. A., Lee G. U., and O'Ferrall, E. C. (**1996**) Covalent attachment of synthetic DNA to self-assembled monolayer films. *Nucleic Acids Res*., 24, 3031-3039.

(29) Lee, G. U., Chrisey, L. A., O'Ferrall, C. E., Pilloff, D. E., Turner, N. H., and Colton R. J. (**1996**) Chemically- specific probes for the atomic force microscope. *Israel J. Chem*. 36, 81-87.

(30) Lamture, J. B., Beattie, K. L., Burke, B. E., Eggers, M. D., Ehrlich, D. J., Fowler, R., Hollis, M. A., Kosicki, B. B., Reich, R. K., and Smith, S. R. (**1994**) Direct detection of nucleic acid hybridization on the surface of a charge coupled device. *Nucleic Acids Res*. 22, 2121-2125.

(31) Vong, T. H., Maat, J., ter van Beek, T. A., van Lagen, B., Giesbers, M., van Hest, J. C. M., and Zuilhof, H. (**2009**) Site-Specific Immobilization of DNA in Glass Microchannels via Photolithography. *Langmuir*, 25, 13952-13958.

(32) Ham, H. O., Liu, Z., Lau, K. H. A., and Messersmith, P. B. (**2011**) Facile DNA immobilization on surfaces through a catecholamine polymer. *Angew. Chem., Int. Ed*. 50, 732-736.

(33) Caipa Campos, M. A., Paulusse, J. M. J., and Zuilhof, H. (**2010**) Functional monolayers on oxide-free silicon surfaces via *thiol-ene* click chemistry. *Chem. Commun*. 46, 5512-5214.

(34) Wong, A. K Y., and Krull, U. J. (**2005**) Surface characterization of 3-glycidoxypropyltrimethoxysilane films on silicon-based substrates. *Anal. Bioanal. Chem*. 383, 187-200.

(35) Tinland, B., Pluen, A., Sturm, J., Weill, G. (**1997**) Persistence Length of Single-Stranded DNA. *Macromolecules* 30, 5763-5765.

(36) Pirrung, M. C. (**2002**) How to Make a DNA Chip. *Angew. Chem., Int. Ed*., 41, 1276-1289.

(37) Twyman, R. M. (**2004**) SNP Discovery and Typing Technologies for Pharmacogenomics. *Curr. Top. Med. Chem.* 4, 1421-1429.

(38) Syvanen, A. C., (**2001**) Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nature Reviews Genetics* 2 (12), 930-942.

(39) Shchepinov, M. S., Case-Green, S. C., and Southern, E. M. (**1997**) Steric factors influencing hybridisation of nucleic acids to oligonucleotides arrays. *Nucleic Acids Res*. 25, 1155-1161.

(40) Lao, A. I. K., Su, X., and Aung, K. M. M. (**2009**) SPR study of DNA hybridization with DNA and PNA probes under stringent conditions. *Biosens. Bioelectron*. 24, 1717-1722.

(41) Morais, S., Marco-Molés, R., Puchades, R., and Maquieira, A. (**2011**) Oxygen Plasma Treated Interactive Polycarbonate DNA Microarraying Platform. *Bioconjugate Chem*. 22, 2573-2580.

(42) Clarke, S. C., (**2001**) Diarrhoeagenic *Escherichia coli*-an emerging problem? *Diagn. Microbiol. Infect. Dis.* 41, 93-98.

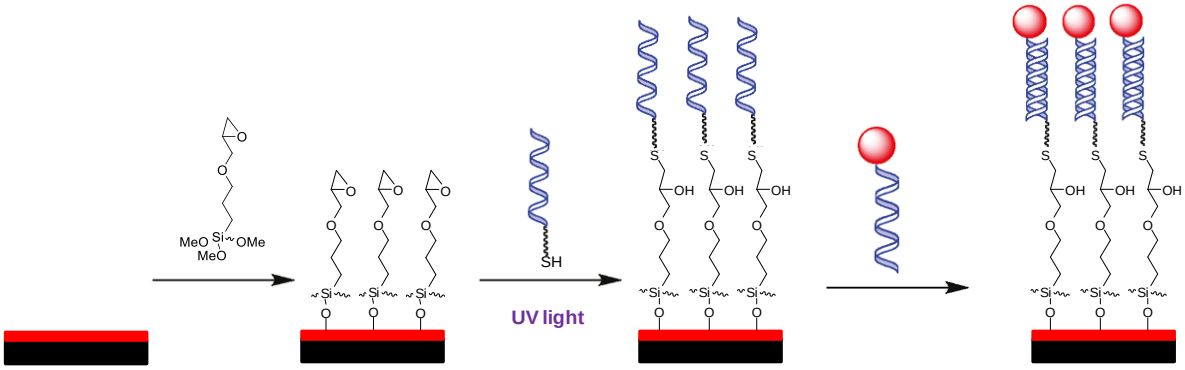

Figure 1. Proposed strategy for DNA immobilization and hybridization.

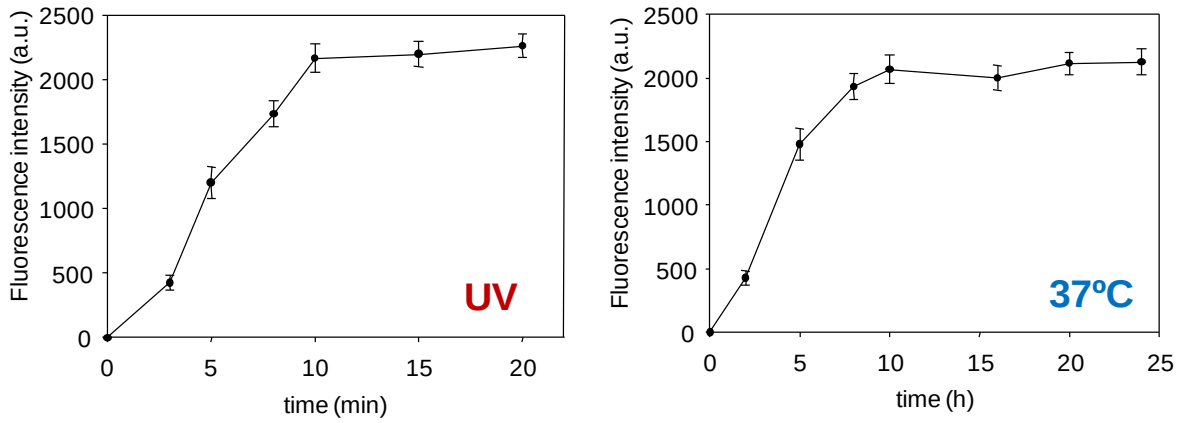

Figure 2. Kinetics of photo (left) and thermal (right) activation of silica surface strategies for oligonucleotide immobilization.

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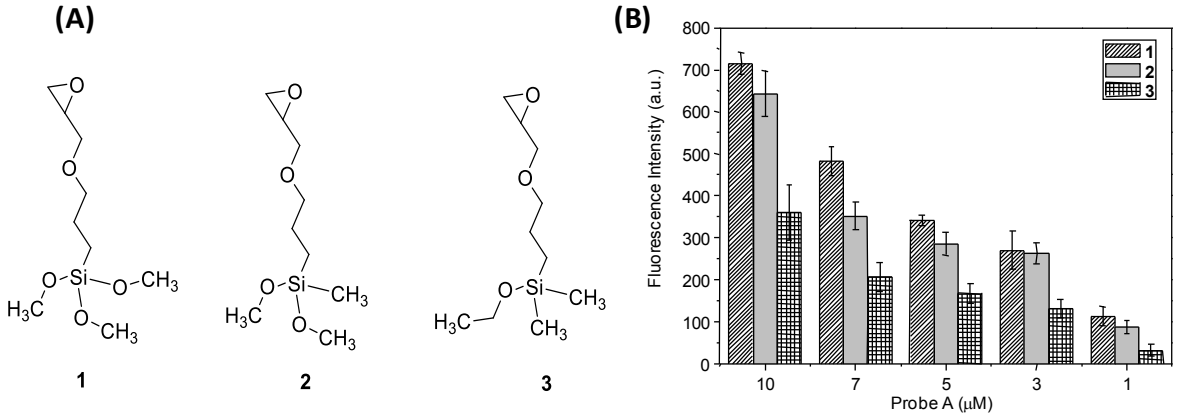

557
558 **Figure 3.** (A) Chemical structures of the assayed organosilane reagents; (B) Effectiveness of each reagent on
559 probe A immobilization.

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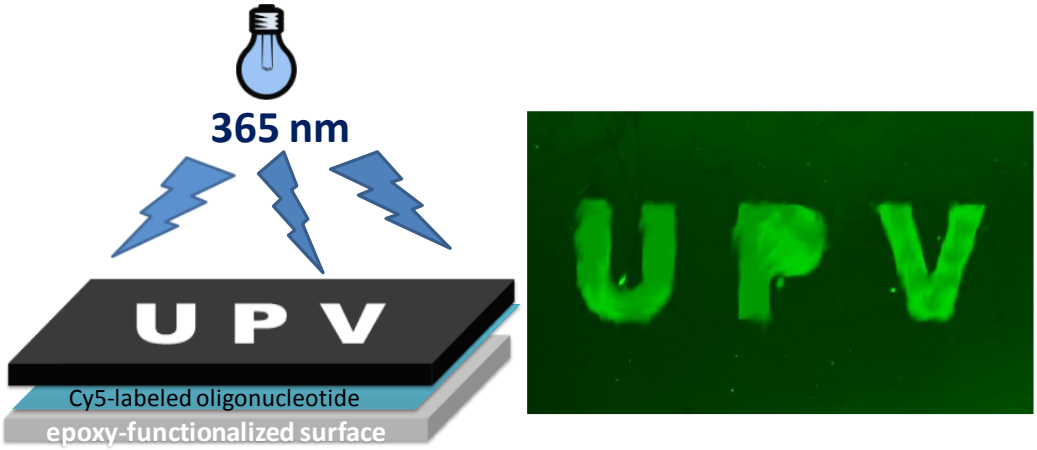

Figure 4. Fluorescence image obtained after irradiation through a photomask.

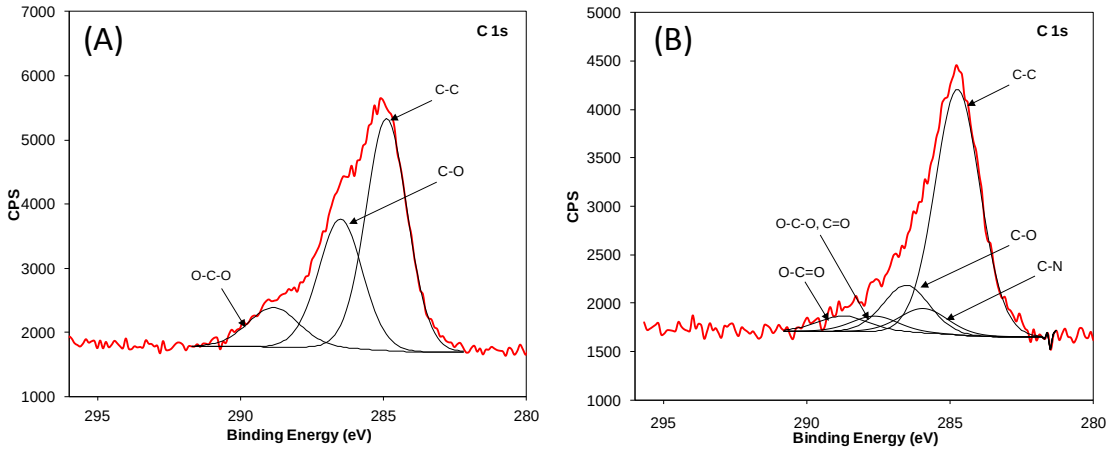

Figure 5. C1s XPS spectra of: (A) epoxy- and (B) DNA-functionalized slide.

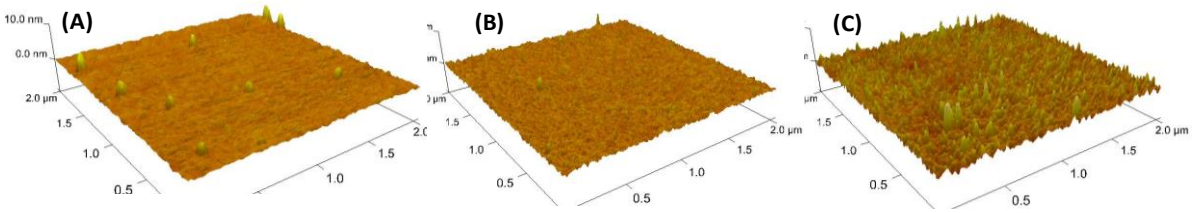

Figure 6. AFM scan of (A) non-functionalized silicon surface, (B) silicon surface coated with GOPTS, (C) silicon surface coated with GOPTS after DNA attachment.

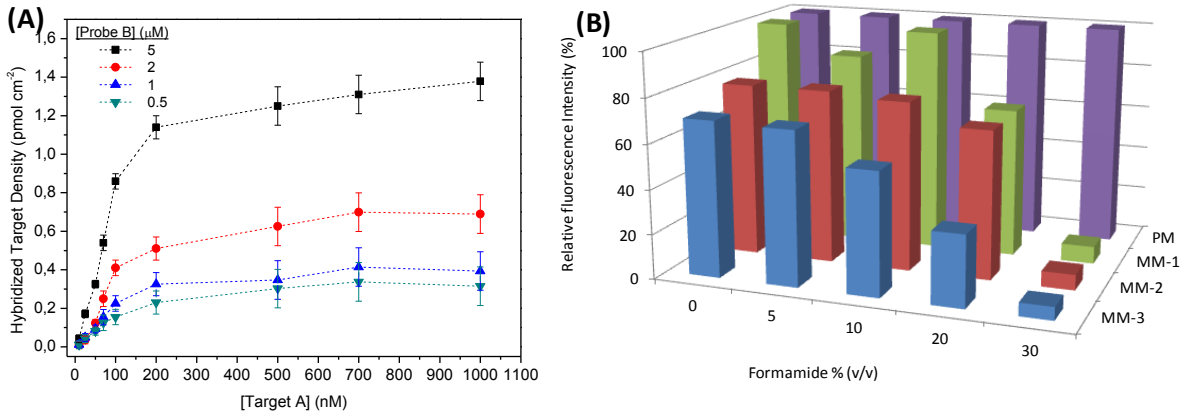

Figure 7. (A) Hybridized target density for different probe B concentrations. (B) Comparison of hybridization efficiency of target with complementary (PM), single-base mismatch (MM-1), two-base mismatch (MM-2) and three-base mismatch (MM-3) probe oligonucleotide.

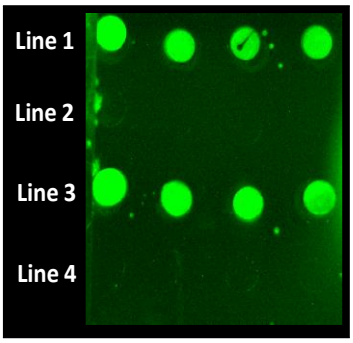

Figure 8. Detection of bacterial *Escherichia coli*. Lines 1 and 3 E. coli probe. Lines 2 and 4 control probe.