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Photoattachment of thiolated DNA probes on SU-8 spin-coated Blu-ray disk surfaces for biosensing†

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A methodology to modify Blu-ray disk (BD) surfaces by spin-coating an SU-8 epoxy photoresist is evaluated, to create a hybrid material with new functionalities. The optical performance of the new film surface disk is analyzed. The light-mediated activation of epoxy-to-thiol chemistry is applied to the site-specific and covalent binding of thiol-ended oligonucleotide probes to the SU-8 disk surface. For the optimized hybridization conditions, a limit of detection of 1 pM is reached employing enzymatic development. PCR products, from a *Salmonella typhimurium* serotype, are detected at an amplicon concentration of 20 pM. The reading of the assays is performed using a commercial BD-player.

Introduction

Biosensing based on a microarray format is a powerful tool in analytical and medical applications. The advantages of such biosensors include miniaturization and assay parallelization, with many uses in fields such as disease diagnosis,[1] gene expression analysis,[2] drug discovery,[3] and food safety[4] or environmental monitoring.[5]

With a focus on nucleic acid determination, the development of microarray methodologies has attracted much attention as it allows rapid, sensitive and selective detection through hybridization procedures.[6] Traditionally, nucleic acid probes have been immobilized on the surface of glass or silane derivatives by physical adsorption or covalent attachment. Covalent anchorage is preferred as higher stability, reproducibility, orientation, and immobilization and hybridization efficiencies are achieved, as well as a lower background signal, as compared to physical adsorption-based approaches.[7]

Alternative to inorganic supports, the use of polymeric materials is spreading given their use as biochip substrates for DNA and protein immobilization, because of biocompatibility, and low cost and mass production. Digital compact disks are an example of how such polymeric platforms show excellent optical properties for analytical applications.[8–11] Besides, commercial digital disk-players may act as microarray readers when positive assays provide a solid deposit on the biorecognition area (*i.e.*, by enzymatic,[12] catalytic[13] or photopolymeric[14] development). BD mainly differs from other digital disks (compact – CD- and digital versatile disks – DVD) as it uses a 405 nm laser beam with a numerical aperture of 0.85, which allows greater information storage than previous devices. In nucleic acid microarray applications, a narrow laser beam should permit the determination of smaller hybridization spots, thus promoting higher probe density per disk and better sensitivity.[15] When considering DNA probe attachment on polymeric surfaces, one major strategy consists of the anchorage of biotinylated oligonucleotides through avidin affinity.[16] However, the protective layer (hardcoat layer), present on commercial BD surfaces to reduce fouling and scratches,[17] reduces the tendency of any biomolecule to be adsorbed on the surface. Thus, modification of the BD surface is necessary for effective and reproducible nucleic acid covalent immobilization. One interesting possibility is to spin coat the disk surface with a polymer to allow further chemical modification to tailor the surface properties (hydrophobicity, linking groups, *etc.*). At the same time, the polymer must maintain the mechanical and optical properties of the disk in order to quantify the assay results using the disk reader. One potential candidate that fulfills all the aforementioned requirements is photoresist SU-8.

SU-8 has been successfully investigated as an active immunoassay surface since it proved to unambiguously retain protein, either directly[18] or after appropriate SU-8 chemical derivatization (aminosililation,[19] coupling with aminated or thiolated crosslinkers, such as glycine and 11-mercaptoundecanoic acid, respectively,[20] treatment with sulfochromic solution[21] or capturing biotinylated biomolecules by avidin, previously anchored to the SU-8 surface[16]). Some publications have generated amine or acidic groups on SU-8 surfaces through the hot-wire vapor deposition of ammonia or under Ar/O_2 plasma, respectively, to covalently attach proteins, including IgGs, by glutaraldehyde-mediated reactions.[22,23] In a recent application,[24] a biophotonic sensor cell for BSA detection has been developed, where the protein is immobilized by a combination of covalent bonds and physical adsorption. However,

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highly sophisticated equipment is required to produce the sensor cell and to obtain signals.

Regarding the linkage of oligonucleotide probes on SU-8 surfaces, several approaches have been developed. Thus, the covalent attachment of amine[25] and phosphoryl[26] oligonucleotides to SU-8 surfaces is described in the literature, although the nature of the bond is not well characterized. Furthermore, the noncovalent attachment of the cholesteryl oligonucleotide has also been reported.[27]

In addition, SU-8-encoded microparticles are chemically modified to perform multiplexed assays[16,28] or to be used for solid-phase synthesis.[24,25,29,30] Recently, a swelling–deswelling method for the surface modification of the 2D and 3D patterns of SU-8 was used, and demonstrated oligonucleotide attachment.[31] However, this method employs toluene that is not applicable to protein attachment due to the risk of denaturation.

Most of the procedures described to immobilize biomolecules on SU-8 surfaces involve incubation times of 1 h or 2 h,[2,6,7,16] the use of harsh chemical treatments (NH_3, plasma or sulfochromic, as mentioned above) and, sometimes, specific detection equipment (CCD cameras, fluorescence scanners or fluorescence confocal microscopes).

Recently, different methods to improve the attachment of thiolated probes onto silicon-based epoxy terminated surfaces were reported. Thus, Mahajan *et al.*[32] described the use of a microwave to cut reaction times; and in our group, a rapid, smart and selective strategy was demonstrated based on light-mediated epoxy opening by thiol ended oligonucleotides.[33] Such a reaction meets most of the requirements stated by Sharpless *et al.*[34] for click chemistry reactions, including very mild reaction conditions, high reaction rates, insensitivity to water and oxygen, atom economy, and the absence of harmful catalysts or solvents.

Thus, the distribution of epoxy SU-8 resin on the surface of BDs may lead to the development of a new hybrid material combining interesting chemical and optical properties: first, the covalent linking of thiolated DNA oligonucleotides in a light-mediated rapid and clean way with good spatial resolution, and second, a high signal-to-noise ratio (sensitivity) is also expected depending on the optical properties of the BD-player laser device.

This article aims, for the first time, at the photoinduced covalent attachment of thiol-modified DNA probes to BD surfaces coated with SU-8. This strategy constitutes a novel, clean and fast methodology to develop microarraying platforms that are applicable to nucleic acid detection. Under the described conditions, oligonucleotides should be successfully immobilized in a spatially controlled manner through selective irradiation. Furthermore, as a proof of concept, the microarrays printed on the new material must be efficiently used for the determination of bacterial *Salmonella typhimurium* DNA.

Experimental

Chemicals

Buffers (printing buffer PBS 1×, prepared from PBS 10×: 0.08 M sodium phosphate dibasic, 0.02 M sodium phosphate monobasic, 1.37 M sodium chloride, 27 M potassium chloride, pH 11; blocking and antibody tracer buffer PBS-T: 10 mM sodium phosphate buffer, 150 mM NaCl, 0.01% (v/v) Tween 20, 5% (v/v) glycerol, pH 7; hybridization buffer SSC 1–4×, prepared from SSC 10×: 0.9 M sodium chloride, 0.09 M sodium citrate, pH 7) and washing solutions were filtered through 0.22 μm pore size nitrocellulose Whatman GmbH membranes (Dassel, Germany) before use.

The SU-8 2000.5 negative photoresist was purchased from MicroChem (Newton, MA, USA). Single-stranded oligonucleotide Probes A, C and E, and Targets C and D were obtained from Sigma-Genosys (Suffolk, UK), while oligonucleotide Probes B and D were acquired from TIB Molbiol Syntheselabor GmbH (Berlin, Germany). Note (Table 1) that some oligonucleotides are terminal-linked to different labels (digoxigenin (DIG) and Cy5 fluorescent dye), while others are thiol end-modified. Acetone of a synthesis grade was purchased from Scharlau (Barcelona, Spain) and 3,3′,5,5′-tetramethylbenzidine (TMB) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The monoclonal anti-digoxigenin antibody [HRP.21H8] (a-DIG/HRP) was purchased from Abcam (Cambridge, UK). Finally, 25 Gbyte BD were ordered from MediaRange GmbH (Bad Soden, Germany). The PCR products from *Salmonella typhimurium* were obtained in our laboratory.

Instruments

A centrifuge from P-Selecta (Barcelona, Spain) was used to spin-coat the SU-8 photoresist on BD surfaces.

The coating conjugates, diluted in printing buffer, were dispensed in a 384-well plate (40 μL per well) and spotted on the disk (20 nL per spot) with a noncontact printing robotic device AD 1500 BioDot Inc. (Irvine, CA, USA) in a microarray format (one to ten matrices of 4 × 1 spots). The reproducibility of the delivered volume was controlled by the steady-state pressure inside the dispensing channel. The working temperature and relative humidity were also controlled (25 °C and 90%, respectively) because these parameters have dramatic effects on the microprinting quality.

Oligonucleotide-Cy5 images were obtained with a home-made surface fluorescence reader device (SFR), described elsewhere.[35]

On the one hand, a 365 nm UV-Vis Vilber Lourmat lamp (Marne la Vallée, France) was employed to irradiate the SU-8

Table 1 Nucleotide sequence of probes and targets used[a]

Name	Sequence (5′ to 3′)
Probe A	Cy5-TTACGATCGACCAGTTAGCCC-T_{15}-SH
Probe B	CCCGATTGATTAGCTAGCATT-Cy5
Probe C	SH-T_{15}-CCCGATTGACCAGCTAGCATT
Probe D	SH-T_{12}-GATTACAGCCGGTGTACGACCCT
Probe E	SH-T_{15}-CGCCGATAACTCTGTCTCTGTA
Target C	Cy5-AATGCTAGCTGG TCAATCGGG
Target D	DIG-AGGGTCGTACACCGGCTGTAATCAAA

[a] Note: DIG stands for digoxigenin. SH refers to thiol-ended modified oligonucleotides.

spin-coated BD surfaces. On the other hand, a 371 nm UV-Vis Jelight Co. Inc. lamp (Irvine, CA, USA) was utilized to irradiate the printed SH-oligonucleotide to achieve covalent binding to the SU-8-modified BD surfaces. Both lamps showed an effective power of 6 W.

The characterization of the modified and unmodified BD surfaces was accomplished with an MZ APO optical microscope (Leica Microsystems GmbH, Wetzlar, Germany), a Veeco Multimode atomic force microscope in the tapping mode (Veeco Instruments Inc., Plainview, NY, USA), and a JSM 5410 scanning electron microscope (JEOL Ltd., Oxford, UK). X-ray photoelectron spectra were recorded using a Sage 150 spectrophotometer from SPECS Surface Nano Analysis GmbH (Berlin, Germany). Nonmonochromatic Al Kα radiation (1486.6 eV) was used as the X-ray source, and operated at a constant pass energy of 30 eV for the elemental specific energy binding analysis. Infrared spectra were obtained using a FT-IR Tensor 27 spectrophotometer from Brucker Optik GmbH (Barcelona, Spain), which incorporated a single reflection diamond ATR accessory or a commercial variable angle reflection unit accessory for IRRAS spectra (2054 scans, incident angle 68°).

A Victor 1420 multilabel counter by Wallac Oy (Turku, Finland) was used to measure the fluorescence of the PCR products, and the concentration was determined from the corresponding calibration curve.

Deposition of SU-8 on BD surfaces

Prior to adding SU-8, the optical active surface of BD was alkali-treated. Briefly, one BD was immersed in a 300 mL of 1 M NaOH solution at 60 °C for 5 min. Afterward, the disk was profusely rinsed with deionized water.

After being dried, the alkali-treated disks were spun at 840 rpm for 1 min to reach a constant angular speed. 1 mL of the SU-8 solution was pipetted near the inner radius of the BD surface and was spun for 1 min to ensure homogeneous polymer distribution. The coated BD was then soft baked at 60 °C for 5 min to remove any remaining solvent contained in the SU-8 polymer. Afterward, the disk surface was irradiated at 365 nm for 1 h. Later, the coated disks underwent a 90 °C post-exposure bake for 10 min before developing the surface with plenty of acetone.

Immobilization of thiol-ended oligonucleotides onto SU-8-coated BD surfaces

Cy5-labeled, thiol-ended oligonucleotides (Probe A) ranging from 20 to 0.5 μM were arrayed (10 × 4 spots) on an SU-8-coated BD surface. Also, Cy5-labeled, unmodified oligonucleotides (Probe B) were printed at 20 μM to control the nonspecific attachment. After printing, the array was irradiated at 371 nm for 5 min in a dark chamber and was rinsed with SSC 1×. Then, the fluorescence signal of the spots was quantified and the amount of immobilized DNA was calculated from the respective calibration curve (performed by spotting known concentrations of Probe A on the surface and registering fluorescence immediately after).

DNA hybridization assays

Thiol-ended oligonucleotides (Probe C) were printed onto the SU-8-coated BD surface at a concentration of 20 μM. The attachment of the probes to the polymeric surface was accomplished as described above. After blocking with 1% casein in PBS for 1 h, the surfaces were rinsed and dried. The arrayed probes were contacted with 0.5–0.1 μM of the Cy5-labeled complementary strand (Target C) at different times (15–90 min), temperature (15–65 °C) and ionic strength (0.117–0.468 M). The results were read with the SFR.

For the hybridization assays employing enzymatic development, thiol-ended oligonucleotides (Probe D) were printed on an SU-8-coated BD surface within the 200 to 10 nM range. After irradiation for 5 min. and washing, the array area was blocked with a 1% (w/v) casein solution for 1 h, and rinsed with PBS-T and deionized water. Bound probes were hybridized with 1–100 nM DIG-labeled complementary strand (Target D) solutions under optimal conditions and were rinsed with SSC 1×. A 1/1000 solution of a-DIG/HRP was used as a tracer to anchor the hybridized complex. Incubation took place at room temperature for 30 min. Afterward, the surface was rinsed with PBS-T and deionized water. Then TMB was added on the arrays which were allowed to react for 8 min at room temperature until a blue precipitate formed on the spots. After rinsing with deionized water, disks were scanned with the BD-player.

Detection of bacterial *Salmonella typhimurium*

BD surfaces were spin coated with SU-8 as described before. Then, solutions containing 200 nM SH-labeled Probe D (*E. coli* specific probe) and 200 nM Probe E (control probe) were spotted onto the functionalized slides creating the microarrays of 2 × 4 spots. Afterward, slides were exposed to UV-light for 5 min and subsequently washed and air-dried. After blocking with casein, DIG-labeled PCR product solutions (50 μL) in hybridization buffer (SSC 1×) were distributed on the chip. PCR duplexes were first melted by 5 min incubation at 95 °C followed by fast cooling for 1 min on ice. After incubating at 37 °C for 1 h, the slides were washed with PBS-T, rinsed with deionized water, and air-dried. The microarray development, using a-DIG/HRP and TMB, and disk reading were performed as aforementioned.

Microarray disk reading and image analysis

A standard BD-player from LG Electronics (Englewoods, NJ, USA) was used as a detector (laser beam $\lambda = 405$ nm). A photosensor (EE-SY125), including a $\lambda = 950$ nm infrared LED (Omron, Scahumburg, IL, USA), was added to trigger data acquisition.

Briefly, the operational principle is based on the detection of the different reflectivity shown by the sensing object and the disk.[8–11] The TMB solid product, generated on appropriately hybridized DNA spots, modified the reflection pattern of the BD surface and attenuated the back-reflected beam intensity that reached the photodiode transducer. It converted reflected light into an analog electrical signal, which was collected and amplified by a custom-built electronic board (DAB). The analog

signals directly acquired from the photodiode transducer correlated well with the optical density of the reaction product; *i.e.*, at the analyte concentration.

The spatial resolution and scanning speed of the BD drive were controlled by the commercial software Nero Disk Speed V4 from Nero Inc. (Glendale, CA, USA), which ran on a Windows-based computer and was connected to the PC by a USB 2.0 universal serial bus interface. The BioDisk custom software was used to control data acquisition (sampling rate and detector gain). The scan began from the inner disk tracks, following the continuous spiral toward outer tracks. The collected data of each detection zone were represented in an ordered, rectangular-shape array, and were stored in independent, uncompressed, binary-format files. This software allows the export of the image in a gray-scale code to a compressed tiff or bitmap format. Then images were processed with the GenePix software (Axon Inst., Union City, CA). Neat signal intensities of each spot were calculated by background subtraction. Note that the background signal was obtained from areas of the analyzed array with no TMB solid product.

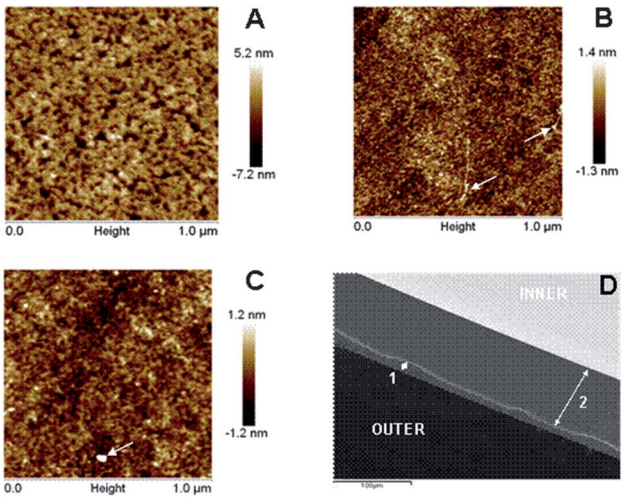

Fig. 1 AFM images corresponding to the bare BD surface (A), the SU-8 spin-coated BD surface (B) and DNA hybridized on the SU-8 BD modified surface (C). Analysis developed on a Veeco Multimode AFM microscope. (D) SEM image corresponding to a cross-section of the BD upper layer film (labeled as 2) spin-coated with SU-8 (labeled as 1).

Results and discussion

BD surface modification by SU-8 spin-coating

One major drawback when attempting to modify BD surfaces lies in their anti-staining, lubricity, scratch resistance and their inertness to any kind of solution, irrespective of the solvent.[17] In fact, from our experience, the chemical composition of the BD optical active surface confers high hydrophobicity, which makes it impossible to obtain a uniform SU-8 film by spin-coating procedures. To allow regular distribution of the photoresist on the disk surface, a treatment used to increase the critical tension (γ_c) of the disk surface was developed. The BD bare surface was highly hydrophobic and presented a contact angle for distilled water of >90°. However, after the alkali treatment, the hydrophilicity of the surface was enhanced, with contact angle values of around 50° (Fig. S1, ESI†). This modification allowed the SU-8 solution to spread uniformly over the entire disk surface. The new surface displayed contact angle values of around 75–80°, which evidenced that the original BD surface was properly modified. Furthermore, the SU-8 spin-coated BD surfaces were assumed stable as the contact angle did not vary over 15 days.

Characterization of SU-8 films

Fig. 1 shows the AFM images corresponding to the common bare BD surface (Fig. 1A), the SU-8 spin-coated BD surface (Fig. 1B) and DNA hybridization accomplished on the SU-8 spin-coated BD surface (Fig. 1C). The image treatment offered the following roughness values: 1.72, 0.391 and 0.394 nm for the bare BD surface and the SU-8 spin-coated BD surface before and after DNA hybridization, respectively. So far, bare BD surfaces show more marked roughness and larger defects than SU-8-modified surfaces, which is in agreement with the planarization process described in the literature.[36] However, small defects could be seen on the spin-coated surface (Fig. 1B, white arrow marks). After DNA hybridization on the SU-8 surface, roughness did not increase, although some buffer salts remaining on the surface could be encountered during the analysis (Fig. 1C, white arrow marks). Thus, more intensive washing was recommended.

Fig. 1D shows a SEM image corresponding to an SU-8 spin-coated BD surface. This image was obtained after the exfoliation of the BD upper layer film. The SU-8 layer (labeled as 1) was seen with an average thickness of around 8 μm. Beneath the photoresist film, it was also possible to observe a bright, wavy thin film associated with the protective polymeric layer present in such disks. In fact, this thin layer showed an average thickness of about 1 μm, which was consistent with the reported values.[14] Furthermore, a thicker layer corresponding to polycarbonate (labeled as 2) was also observed, with an average thickness of 86 μm. As before, this value falls within the range of the technical values offered by most BD manufacturers (a maximum of 100 μm). Moreover, the SU-8 inner side seemed to rest on a fluctuant surface as deeper and irregular SU-8 deposit areas are visualized. Conversely, the SU-8 external surface appeared flatter and smoother. This behavior was in accordance with previous AFM results, where the bare BD showed more marked roughness than the SU-8 cured one.

An XPS analysis of bare BD and cured SU-8 spin-coated surfaces was also performed (Fig. S2, ESI†). It was possible to observe how the deposition of the SU-8 thin layer on top of the BD platform reduced the signal associated with Si 2s and Si 2p peaks, and completely erased the signal attributed to the N 1s peak. Silicon was incorporated into the hardcoat BD upper layer formulation (probably as inorganic microparticles) in order to increase wear resistance, while nitrogen formed part of the polymeric hardcoat matrix (*e.g.*, amide bonds). Neither silicon nor nitrogen was present in the SU-8 mixture. For this reason, the aforementioned reductions in signal intensity were attributed to the described surface modification.

As with the oxygen chemical surface composition, when the O 1s peak was deconvoluted (Fig. S2B and C, ESI†), no ester contribution (O–C═O, 533.7 eV) was observed after SU-8 deposition as compared to the bare BD surface. Ester is a common functional group present in hardcoat formulations.[17]

Regarding the deconvolution of the C 1s peak (Fig. S2D and E,† ESI), the SU-8-coated surface showed the contribution assigned to aromatic carbon (C–C, 284.7 eV), which was absent in the bare BD. This observation was consistent with the presence of an SU-8 film on top of the BD surface, as would emerge from the benzyl rings located in the SU-8 structure. Furthermore, the carbon percentage calculated on the bare BD surface was 68.6%, while a value of 82.6% was obtained after SU-8 deposition. The surface enrichment on this element was assumed given the carbon content in the SU-8 matrix.

From the XPS data reported herein, it was possible to unambiguously confirm the existence of an SU-8 layer on top of the BD platform.

The ATR FT-IR spectra, corresponding to a commercial BD surface before and after SU-8 photoresist spin-coating, were recorded. The as-labeled BD/SU-8 spectrum was the result of combined contributions related to the absorptions taking place in both the upper BD layers and the SU-8 thin film. Using a raw BD as reference, it was possible to obtain the absorbance contributions ascribed to the SU-8 thin film (Fig. S3, ESI†). Some characteristic SU-8 peaks were identified at 3050, 2930, 2830, 1600–1500 and 850–600 cm^{-1}, and were associated with C–H stretching (contribution of cyclic ether and tensions in the aromatic ring), $–CH_3$, $–CH_2–$, phenyl nucleus and aromatic C–H bending, respectively. These results supported, together with the XPS data shown, that the chemical composition of the upper layer observed by SEM is due to the SU-8 coating.

Probe immobilization on SU-8 spin-coated BD surfaces

Coating the BD with SU-8 provided glycidyl moieties on the surface. Such epoxy groups can undergo ring opening with amine or thiol nucleophiles and this type of reaction has been already used in the literature for the probe attachment onto SU-8 surfaces.[26] Also, the SU-8 resin presents a high ability to immobilize probes by adsorption. The nucleophilic attack requires long incubation times with the probes. However, it can be accelerated by raising the temperature[37] or by microwave irradiation.[32] We recently demonstrated an alternative method to induce a rapid thiol-epoxy coupling reaction by photoactivation, used to develop microarrays on silicon-based surfaces.[33] Light-induced immobilization presented the advantage against the abovementioned strategies that spatial discrimination was possible, for example, using a photomask. Thus, irradiation with UV-light at 371 nm was employed to induce the thiolated probe attachment onto the epoxy groups of SU-8 coated BD.

Thiol-ended, Cy5-labeled oligonucleotides (Probe A) were arrayed at different concentrations, ranging from 0.5 μM to 20 μM, onto SU-8 coated BD as described in the Experimental section. After irradiation for 5 minutes and washing the microarray fluorescence was registered and quantified, and compared with a parallel assay where the same oligonucleotide solutions were printed onto the SU-8 surface and kept for 5 minutes in the dark, and washed and measured. The comparison revealed the role of light in the probe attachment process, and thus the fluorescence intensities for 10 μM of spotted probe were 1189 ± 208 a.u. (SNR 12) and 12 832 ± 1822 a.u. (SNR 100) for the non-irradiated and irradiated arrays, respectively. Even more, when the fluorescence of Probe A irradiated array was compared with the fluorescence obtained for the same array incubated in a dark, humid chamber overnight and with an array of non-thiolated oligonucleotides (Probe B) irradiated for 5 minutes, the fluorescence for the first case was significantly higher than the fluorescence obtained for the others (Fig. 2(a) and (b)). This confirmed the necessity of both the irradiation and the thiol group to achieve an effective attachment in short times. The role of epoxy moieties in the probe attachment was demonstrated using an SU-8 BD where the epoxy rings were opened with an acidic treatment (3 M H_2SO_4 for 90 minutes) before printing the array of thiolated probes. As expected, the fluorescence intensity for this array was in the range of that obtained for the nonthiolated oligonucleotide. Finally, the thermal stability of the attached probes was tested by heating for 10 minutes at 95 °C, a fluorescence loss of 6% was observed, in accordance with data found in the literature.[26] Thus, all the observations pointed to a covalent attachment of the probes, providing thus a robust link in very short times. Furthermore, photoactivation allowed the selective bonding of thiolated oligonucleotides on SU-8 BD surfaces, which implies potential applications where spatial resolution is required; for instance, it is especially interesting when developing micro- and nano-

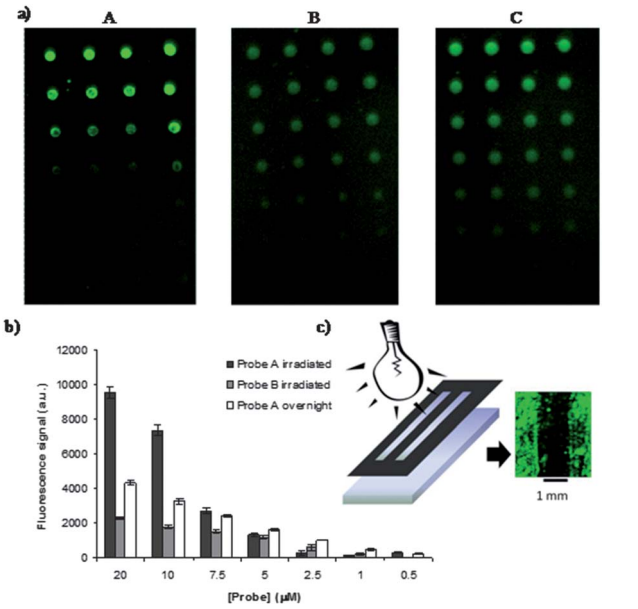

Fig. 2 (a) Microarray fluorescence images of Probe A (A) and Probe B (B) irradiated at 371 nm for 5 min, and Probe A incubated in the dark overnight (C). Probe concentrations: 20–10–7.5–5–2.5–1–0.5 μM (from top to bottom), (b) comparison of quantified fluorescence values obtained for the three microarrays, (c) details of the fluorescence image obtained after irradiation through a photomask.

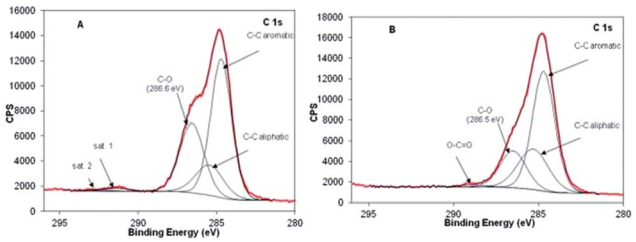

Fig. 3 C 1s XPS spectra of SU-8 BD (A) and DNA-functionalized (B) surfaces.

biosensors, where a selective attachment of the bioreceptors on the sensing area is of outmost importance to reach optimal sensitivity.[38] To demonstrate the patterning of surfaces, Probe A at 10 μM in PBS was spread out onto the SU-8 BD surface, allowed to dry and covered with a photomask. After irradiation for 5 minutes and washing, the surface was read with the SFR (Fig. 2(c)). Thus, selective immobilization of DNA probe was achieved by means of UV irradiation in a simple, clean, fast, and efficient way. Although previous strategies describing the covalent immobilization of thiolated oligonucleotides onto SU-8 functionalized surfaces have been described by conventional nucleophile attack, to our knowledge this is the first time that light-induced selective immobilization has been reported employing these reagents.

The DNA modified surface was characterized using WCA, XPS and IRRAS. WCA diminished from 75 to 80° of the SU-8 BD surface to 53° after probe attachment, with the last value being in agreement with data reported in the literature.[33,39]

The XPS analysis also showed changes in the surface composition of the SU-8 BD surface after DNA attachment (Fig. 3). For the DNA-functionalized surface, the C 1s spectrum was resolved into four different carbon positions with varying intensities: C–C aromatic (284.7 eV), C–C aliphatic (285.3 eV), C–N and C–O (286.5–286.8 eV), and N–C(=O)–N (288.9 eV).[40] The relative concentrations of the different carbon species indicated that after DNA attachment there was an increase in C–C aliphatic contribution and a decrease in C–C aromatic contribution.

Finally, the IRRAS spectrum, which was measured taking the raw SU-8 BD surface as reference, showed a broad band around 1700 cm^{-1} corresponding to carboxylic contributions attributed to DNA (Fig. S4, ESI†).[41]

In conclusion, the effective light-mediated anchorage of thiolated probes to the epoxy moieties of SU-8 by the formation of a robust covalent bond was demonstrated.

Hybridization of DNA probes chemically bound to SU-8

Time, temperature and ionic strength were studied to optimize the hybridization conditions for a thiolated DNA probe and its complementary Cy5-tagged strand (Probe C and Target C). Fig. 4 depicts the fluorescence from a hybridization of 20 μM of Probe C and 0.5 (or 0.1 μM) of Target C. Both concentrations were previously adjusted to obtain the strongest nonsaturated signal on the BD-player. The optimal system conditions were obtained at 30 min, 37 °C and 0.234 M ionic strength.

Fig. 4A shows the fluorescence signal measured on hybridized arrays for different incubation times ranging from 15 to 90 min. In this study, temperature and ionic strength were kept constant at 37 °C and 0.234 M, respectively. When hybridization was assayed for 15 min, the resultant fluorescence curve displayed the lowest value in the series. For a hybridization time of 30 min, a signal increase of over 200% as compared with the 15 min assay took place. However, longer hybridization times did not significantly increase the fluorescence signal, with values of 9% after 60 min and 7% after 90 min as compared to the 30 minute assay. Thus, beyond 30 min, hybridization saturation was achieved irrespective of incubation time, indicating that every accessible probe coated on the surface was already bound to a DNA target. So carrying out the hybridization step for 30 min was established.

Fig. 4B shows the fluorescence hybridization curves for different temperatures ranging from 15 to 65 °C. The data shown correspond to a hybridization achieved with probe and target concentrations of 20 and 0.1 μM, respectively, and an incubation period of 30 min, and an optimum hybridization value was achieved at 37 °C. This value is coherent with the

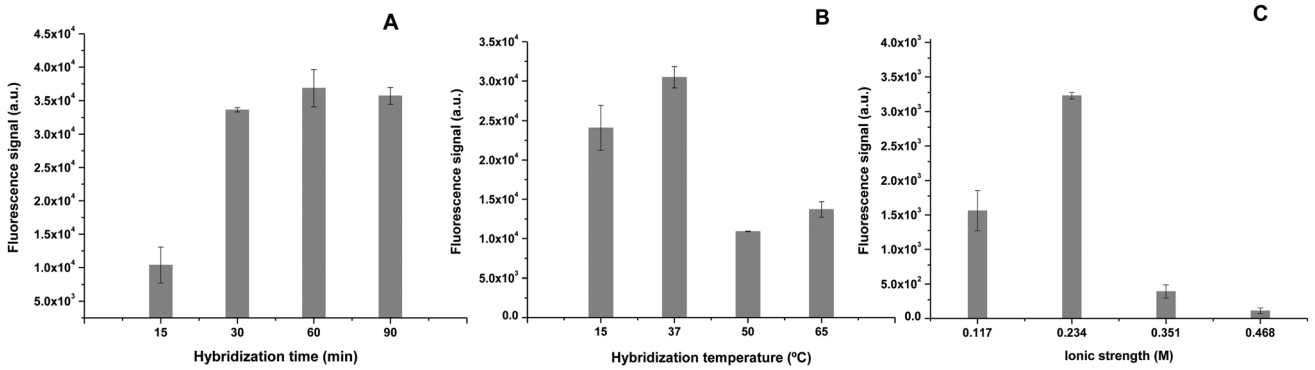

Fig. 4 Optimization of the hybridization time (A) Probe C and Target C concentrations were 20 μM and 0.5 μM, respectively. Experiments carried out at 37 °C and 0.234 M (ionic strength). Optimization of the hybridization temperature (B) probe and target concentrations were 20 μM and 0.1 μM, respectively. Experiments carried out for 30 min and 0.234 M (ionic strength). Optimization of the hybridization ionic strength (C) probe and target concentrations 20 μM and 0.1 μM, respectively. Experiments carried out at 37 °C for 30 min.

hybridization tendencies described in the literature, which depends on the base nature of the implicated oligonucleotides and, therefore, on their melting temperature: a low temperature value may not suffice to promote oligonucleotides hybridization, while a high value may force complementary strands to remain apart due to excess kinetic energy.

The influence of ionic strength on the hybridization step is shown in Fig. 4C. The displayed data correspond to an ionic strength range of 0.117–0.468 M, a probe concentration of 20 μM, a target concentration of 0.1 μM, a time of 30 min and a temperature of 37 °C. As previously described for the hybridization temperature, a curve with a maximum fluorescence value was obtained. Here, an ionic strength of around 0.234 M was considered the optimum value for the hybridization of the proposed probe/target oligonucleotide system. For the current probe/target DNA system, the aforementioned ionic strength value contained a concentration of cationic species that balanced between DNA strand repulsion and hybridization events. Raising the salt concentration may cause the repulsion due to excessive positive charge on the DNA duplex, which, in turn, may affect the hybridization.

DNA hybridization quantified by a BD-player

Having optimized the hybridization process, a step forward was taken in order to employ a BD-player for the assay read-out and to work out the achievable limit of detection when DNA hybridization was enzymatic developed on SU-8 spin-coated BD surfaces. For this purpose, the interaction of a thiolated DNA probe with its complementary DIG-tagged strand (Probe D/Target D) was studied using a-DIG/HRP as a tracer and TMB as the developing solution.[8–10] Note that both Probe D and Probe C have 36 similar sequences of bases (18T, 6G, 5A and 7C for Probe D and 20T, 4G, 5A and 7C for Probe C) and a molecular weight close to 11.5 kDa. By assuming that both oligonucleotides would behave the same way, Probe C hybridizing optimal conditions were applied to the Probe D assays. The signal generated by the enzymatic reaction on the BD/SU-8 surfaces was recorded with the BD-player device prototype.

The images corresponding to the assayed arrays (Fig. S5, ESI†) allowed us to observe how the hybridization signal intensity increases with the thiolated probe concentration. Bound probes frequently reach an optimal surface density, beyond which the developed signal remains stable (plateau, due to saturation) or diminishes (due to steric crowding). In our case, the highest probe concentration assayed (200 nM) did not suffice to reach the plateau in the current sensing platform. However, it was of no interest to print higher probe concentrations as the HRP-mediated solid product might over-saturate the BD-player signal, which would hinder adequate surface analyses.

The reason for this high probe concentration "tolerance" was related to the density of the epoxy groups located on the SU-8 film surface. A qualitative comparison was made between physically streptavidin-mediated oligonucleotide adsorption and SH-oligonucleotide covalently bound to SU-8 systems. Accordingly, when assuming a packed square adsorption format for streptavidin (SA), the surface occupancy for this protein has been referred to as 25 nm^2 in the literature.[42] For the SU-8 surface, a ratio of 2.7 epoxy units per nm^2 was calculated using licensed Chem3D ultra software. This means 68 epoxy groups covering the same surface as one SA molecule. Thus, the surface probe loading capacity is higher and then SU-8 modified surfaces could promote higher hybridization yields than biotinylated oligonucleotide/SA systems, although the maximum hybridization yield would be limited by the crowding effects of immobilized probes.

With the same probe concentration printed in different arrays, the signals generated by the BD-player remained more or less constant when lowering the target concentration from 100 to 1 nM (signal 10 000 a.u.). Then a rapid decrease, *i.e.*, a steep slope, was observed when lower target concentrations were used to hybridize with the probe. For these conditions, a target concentration of at least 1 pM was certainly distinguishable from the background signal and from the negative hybridization control (Probe E mean value plus 3 times its standard deviation). These results confirmed that hybridization could be successfully accomplished by following the proposed approach with sensitivity in the picomolar order, which highlights this platform as an interesting candidate for DNA determination applications.

Inter- and intradisk reproducibility experiments (three replicates each) were accomplished by allowing the hybridization between 50 nM of the printed Probe D with 0.1 nM of Target D under the previously optimized conditions. Intradisk and interdisk reproducibility of 6% and 14% were achieved, respectively.

Detection of *Salmonella typhimurium* DNA

Finally, the use of the developed surfaces as microarray platforms for real applications was studied. For this purpose, the PCR-amplified DNA products from pathogen *Salmonella typhimurium* were sensitively and selectively detected. Although most *S. typhimurium* strains are harmless, some serotypes are pathogenic and can cause serious food poisoning in humans. So point-of-care methodologies to allow the rapid, selective and sensitive detection of such pathogens are of much interest. The nucleotide sequence of immobilized Probe D was complementary to the central region of a 152-base amplicon specific to detect an innocuous serotype of *S. typhimurium*. Thus, 200 nM of Probe D and Probes B and E, as controls, were immobilized on the SU-8-modified BD surface following the aforementioned light-mediated procedure. The PCR product concentration was determined by a fluorescence measurement and was 1.51 nM. In order to apply the enzymatic development methodology proposed above, deoxyribonucleotides tagged with digoxigenin were added to the PCR mixture. So, the amplified DNA strands incorporated digoxigenin molecules into their structure. The main results are shown in Fig. 5.

A range from 1/20 to 1/200 of amplicon dilution factors was studied for the hybridization assays. The microarrays containing a dilution as low as 1/80, which corresponds to 20 pM of amplicon, showed a BD-player signal after a-DIG/HRP tracer

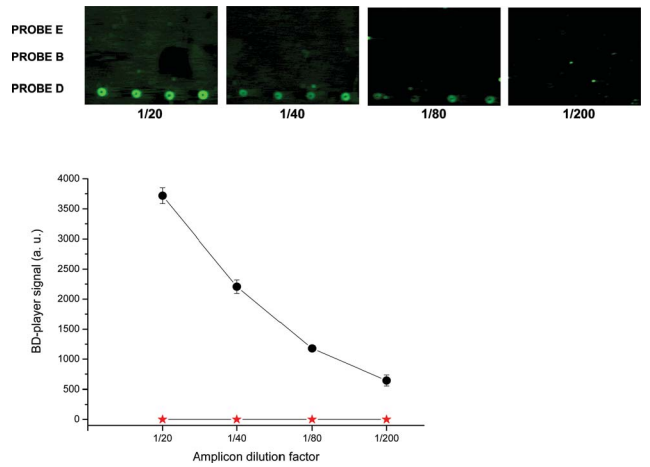

Fig. 5 Top images show the sequence of assayed areas as captured by the BD-player device (type of probe appears on the left and values beneath each image refer to target amplicon dilutions). Graph reflects the BD-player signal corresponding to the hybridization of Probe D with *S. typhimurium* PCR product (37 °C, 30 min 0.234 M in SSC 2×). Hybridization negative control coated at 200 nM (red star).

development with TMB. Samples more diluted than 1/80 displayed no confident values. Note that a sensitivity of 1 pM was achieved after the hybridization of Probe D and Target D. However, the use of amplicon as a target lowered sensitivity to 20 pM. Such behavior was consistent with a higher hindrance and disturbances occurring when longer DNA strands are involved (152 bases in the amplicon *versus* 26 bases in Target D). Noncomplementary hybridization control spots generated no quantifiable signal. Recently, similar systems based on hybridization events developed by an enzymatic reaction were reported. Although comparable in order, our results revealed an improvement in the limit of detection to any of the previously mentioned examples; *i.e.*, the hybridization carried out on SU-8 coated-glass surfaces[43] and on DVDs,[10] where the lowest concentrations of target detected were 250 and 350 pM, respectively. In the latter case, improvement in sensitivity was attributed to the combination of the high performance of the SU-8 surface and the use of Blu-ray reading technology, which achieved a better resolution than the DVD. Furthermore, the strategy described in the current paper has the added value of presenting a very fast, clean immobilization approach for DNA probe attachment.

Conclusions

A protocol to transform the inert surface of a commercial BD into a chemically active platform for microarray probe immobilization has been developed. This approach does not influence optical BD-player device performance, and the complete scan of modified disk surfaces is successfully achieved.

The proposed epoxy surfaces have been demonstrated to selectively and covalently bind thiolated DNA probes and to recognize their complementary strand with specificity. The combination of BD technology and light-induced probe immobilization helps accomplish high sensitivity that is comparable to the results reported for previous systems based on enzymatic development.

The use of SU-8 on BD opens a way to construct low-cost high-density microarrays[44] on the disk surface by selective irradiation.

Finally, the described methodology appears to be a tool to generate competitive microarray platforms onto BD surfaces. Furthermore, the use of the BD-player device as a microarray detector settles the potential implementation of the developed procedure in point-of-need DNA analyses of real samples, as demonstrated herein for the sensitive detection of *Salmonella typhimurium* PCR products.

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