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

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Abstract
A methodology to modify Blu-ray disk (BD) surfaces by spin-coating of an SU-8 epoxy photoresist is evaluated and the optic performance of the new film surface disk is analyzed. The light-mediated activation of epoxy-to-thiol chemistry was applied to the site-specific and covalent binding of thiol-ended oligonucleotides to the SU-8 disk surface. Hybridization conditions for the attached probe and its full complementary target were optimized, and a limit of detection of 1 pM was reached by employing enzymatic development. Testing PCR products, from an innocuous *Salmonella typhimurium* serotype, detected amplicon concentrations of 20 pM. The reading of the assays was performed using a commercial BD-player.

Keywords: SU-8, Blu-ray disk, epoxy-to-thiol, hybridization, DNA sensing, microarray

1. Introduction

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

With a focus on nucleic acids 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, degree of immobilization and hybridization efficiency are achieved, while a background signal lower as compared to physical adsorption-based approaches [7].

Alternatively to inorganic supports, the use of polymeric materials is spreading given their use as biochip substrates for DNA and protein immobilization, and because of biocompatibility, low cost and mass production. Digital compact disks are an example of how such polymeric platforms show excellent optical properties for analytical applications [8,9]. Besides, commercial digital disk-players may act as detectors when assays are accomplished by the formation of a solid deposit on the biorecognized area (i.e., by enzymatic [10], catalytic [11] or photopolymeric [12] procedures). BD mainly differs from other digital disks (compact -CD- and digital versatile disks -DVD) as it uses a 405 nm laser beam with a numeric 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.

When considering a DNA probe attachment on polymeric surfaces, one major strategy consists in the anchorage of biotinylated oligonucleotides through avidin affinity [13]. However, the protective layer (hardcoat layer), present on commercial BD surfaces to reduce foul and scratches [14] 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 acids covalent immobilization. One interesting possibility is to spin coat the disk surface with a polymer to allow the 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 [15] or after appropriate SU-8 chemical derivatization (aminosililation [16], coupling with thiolated crosslinkers, such as glycine and 11-mercaptoundecanoic acid [17], treatment with sulphocromic solution [18] or capturing biotinylated biomolecules by avidin, previously anchored to the SU-8 surface [13]). 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, in order to covalently attach proteins and IgGs through glutaraldehyde-mediated reactions [19-20]. In a recent application, a biophotonic sensor cell for BSA detection has been developed, where the protein is immobilized by a mixture of covalent and physical adsorptions [21]. However, highly sophisticated equipments are 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 oligonucleotides to the epoxy functions on SU-8 surfaces by incubation at 37ºC [22] or 45ºC when considering amine and phosphoryl oligonucleotides [23] is described in the literature. Furthermore, the non covalent attachment of the cholesteril oligonucleotide has been reported [24].

In addition, SU-8-encoded microparticles are chemically modified to perform multiplexed assays [13,25] or to be used for solid-phase synthesis [21-22,26-27]. Recently, a swelling-deswelling method for the surface modification of the 2D and 3D patterns of SU-8 was described, and demonstrated oligonucleotide attachment [28]. However, this method involves the use of toluene, and 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 [2,6,7,13], the use of harsh chemical treatments (NH_3, plasma or sulphocromic, as mentioned above) and, sometimes, specific detection equipment. For instance, all the references reporting results are based on the fluorescence images obtained with CCD cameras, fluorescence scanners or fluorescence (confocal) microscopes.

Recently, different methods to improve the attachment of thiolated probes onto silicon-based epoxy ended surfaces were reported. Mahajan *et al.* [29] described the use of a microwave to cut reaction times. In our group, a rapid, smart and selective strategy has been established based on light-mediated epoxy opening by thiolated DNA oligonucleotides [30]. Such a reaction meets most of the requirements stated by Sharpless *et al.* [31] 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 covalent linking of thiolated DNA oligonucleotides in a light-mediated rapid and clean way with good spatial resolution. A high signal-to-noise ratio (sensitivity) is also expected depending on the BD-player laser device optical properties.

This article demonstrates, for the first time, 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 have been successfully immobilized in a spatially controlled manner through selective irradiation. Furthermore, the constructed microarrays efficiently allowed the determination of bacterial *Salmonella typhimurium* DNA.

2. Experimental procedures

2.1. Materials

Buffers (printing buffer PBS 1x, prepared from PBS 10x: 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: 10mM sodium phosphate buffer, 150mM NaCl, 0.01% (v/v) Tween 20, 5% (v/v) glycerol, pH 7; hybridization buffer SSC 1-4x, prepared from SSC 10x: 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 oligonucleotides PAT2, PAT3, SYM5, SYM15, SYM43, SYM44, and SYM50 were obtained from Sigma-Genosys (Suffolk, UK), while oligonucleotides PAT16 and SYM25 were acquired from TIB Molbiol Syntheselabo 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 antiboby [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.

TABLE 1

2.2. Characterization techniques and instrumentation

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/well) and spotted on the disk (20 nL/spot) with a noncontact printing robotic device AD 1500 BioDot Inc. (Irvine, CA, USA) in a microarray format (one to ten matrices of 4x1 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 printed microarray quality.

Oligonucleotides-Cy5 images were obtained with the homemade surface fluorescence reader device (SFR), described elsewhere [32].

On the one hand, a 365 nm UV-VIS Vilber Lourmat lamp (Marne la Vallée, France) was employed to irradiate the SU-8 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 value 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 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, and operated at a 30 eV constant pass energy for the elemental specific energy binding analysis. The vacuum in the spectrometer chamber was 9×10^{-9} hPa and the sample area analyzed was 1 mm^2. Infrared spectra were obtained with a FT-IR Tensor 27 spectrophotometer from Brucker Optik GmbH (Barcelona, Spain), which incorporated a single reflection diamond ATR accessory.

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.

2.3. Spin-coating procedure

Prior to adding SU-8, the optical active surface of BD was alkali-treated. Briefly, one BD was immersed in a 300 mL 1 M NaOH solution at 60ºC 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 using a laboratory centrifuge (Section 2.2). While rotating at 840 rpm, 1 mL of the SU-8 solution was dispensed with a pipette near the inner radius of the BD surface and was spun for 1 min to ensure that the polymer solution was evenly distributed over the entire disk surface. The coated BD was then soft baked at 60ºC for 5 min to remove any remaining solvent contained in the SU-8 polymer. Afterward, the coated surface was irradiated at 365 nm for 1 h. Later, the coated disks underwent a 90ºC post-exposure bake for 10 min before developing the surface with plenty of acetone.

2.4. DNA assays

2.4.1. Covalent binding of thiolated oligonucleotides to SU-8-coated BD surfaces

SH-oligonucleotide-Cy5 (SYM44) was arrayed on an SU-8-coated BD surface ranging from 20 to 0.5 μM. Oligonucleotide-Cy5 (SYM41) was printed at 5 μM to control the non specific attachment. After printing, the array was irradiated at 371 nm for longer times (0-30 min) in a dark chamber and was rinsed with SSC 1x. 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 SYM44 on the surface and registering fluorescence immediately after). In order to discard the non specific attachment of the thiolated oligonucleotide to the polymeric surface, fluorescence from a non irradiated SYM44 array deposited on an SU-8-coated BD surface was also analyzed.

2.4.2. Optimization of the DNA hybridization conditions

SH-oligonucleotide (SYM43) was printed on an SU-8-coated BD surface at a concentration of 20 μM. The covalent attachment of the probes to the polymeric surface was accomplished as described in Section 2.4.1. In order to optimize the hybridization step, the arrayed probes were contacted with 0.5-0.1 μM of complementary oligonucleotide-Cy5 (SYM5) at different times (15-90 min), temperature (15-65ºC) and ionic strength (0.117-0.468 M).

2.4.3. Enzymatic development of covalently bonded probes

SH-oligonucleotide (PAT16) was printed on an SU-8-coated BD surface within the 200 to 10 nM range. The covalent attachment of the probes to the polymeric surface was accomplished by irradiation for 5 min, as described in Section 2.4.1. The array area was blocked with a 1% (wt/vol) casein solution for 1 hour and was rinsed with PBS-T and deionized water. Bound probes were hybridized with 1-100 nM oligonucleotide-DIG (SYM5) solutions under optimal conditions (as described in Section 3.4) and were rinsed with SSC 1x. 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.

2.5. Disk detection and image analysis systems

A standard BD-player from LG Electronics (Englewoods, NJ, USA) was used as a detector (laser beam λ=405 nm). A photosensor (EE-SY125), including a λ=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 [8,9]. 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.

3. Results and discussion

3.1. 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, irrespectively of the solvent [14]. 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 the photoresist regular distribution 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 > 90º. However, after the alkali treatment (Section 2.3), the hydrophylicity of the surface was enhanced, with contact angle values of around 50º. This modification allows the SU-8 solution to spread uniformly over the entire disk surface. The new surface displayed contact angle values of around 75-80º, which evidences 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.

3.2. Characterization of SU-8 films

Figure 1 shows the AFM images corresponding to common bare BD surface (Fig. 1A), the SU-8 spin-coated BD surface (Fig. 1B) and DNA hybridization accomplished on SU-8 spin-coated BD surfaces (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 [33]. However, small defects can be seen on the spin-coated surface (Figure 1B, white arrow marks). After DNA hybridization on the SU-8 surface, roughness did not increase, although some buffer salts remaining on the surface can be encountered during the analysis (Fig. 1C, white arrow marks). Thus, more intensive washing is recommended.

FIGURE 1

Figure 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) is seen with an average thickness of around 8 μm. Beneath the photoresist film, it is also possible to observe a bright, wavy thin film associated with the protective polymeric layer present in such disks (Section 3.1). In fact, this thin layer shows an average thickness of about 1 μm, which is consistent with reported values [14]. Furthermore, a thicker layer corresponding to polycarbonate (labeled as 2) is also observed, with an average 86 μm thickness. As before, this value falls within the range of the technical value offered by most BD manufacturers (a maximum of 100 μm). Moreover, the SU-8 inner side seems to rest on a fluctuant surface as deeper and irregular SU-8 deposit areas are visualized. Conversely, the SU-8 external surface appears flatter and smoother. This behavior is 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 (Figure S1, ESI). It is possible to observe how the deposition of the SU-8 thin layer on top of the BD platform reduces the signal associated with peaks Si 2s and Si 2p, and completely erases 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 (Figure S1 B and C, ESI), no ester contribution (O-C=O, 533.7 eV) is observed after SU-8 deposition as compared to the bare BD surface. Ester is a common functional group present in hardcoat formulations [14].

Regarding the deconvolution of the C 1s peak (Figure S1 D and E, ESI), the SU-8-coated surface shows the contribution assigned to aromatic carbon (C-C, 284.7 eV), which is absent in the bare BD. This observation is 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 is assumed given the carbon content in the SU-8 matrix.

From the XPS data reported herein, it is 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 (Figure S2 A, ESI). Upon first glance, both spectra are similar. This is due to the deep penetration of the infrared beam going through the SU-8 thin film and reaching the polymeric layers located underneath, which constitute the BD platform. So, the as-labeled BD/SU-8 spectrum would be the result of the combined contributions related to the absorptions taking place in both the upper BD layers and the SU-8 thin film. By subtracting the spectra (BD/SU-8 from the bare BD), it was possible to obtain the absorbance contributions ascribed to the SU-8 thin film (Figure S2 B, 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 confirm, together with the XPS data shown, that the chemical composition of the upper layer observed by SEM is due to the SU-8 coating.

3.3. Covalent DNA probe attachment on SU-8 spin-coated BD surfaces

The potential application of the light-induced thiol-epoxy coupling chemistry for creating microarrays onto the SU-8 modified BD was then considered.

It was crucial to determine if the reaction would be exclusively activated by light or if non specific interactions should be considered. SH-oligonucleotide-Cy5 (SYM44) was printed at the 10 μM concentration and was irradiated as previously described (Section 2.4.1). In a parallel assay, the same oligonucleotide solution was printed in the nearby region onto the SU-8 surface and was kept in the darkness for an equivalent period of time. Both arrays were rinsed with SSC 0.1x and distilled water before measuring their fluorescence signals (Figure S3, ESI). Processed data offered the following values for fluorescence intensities: 1189±208 a.u. and 12832±1822 a.u. for the non irradiated (Fig. 4A) and irradiated (Fig. 4B) arrays, respectively. Thus, when the thiolated oligonucleotides arrays, within the studied range of concentrations, were irradiated on an SU-8 surface, a neat 11-fold greater signal was obtained if compared with the non irradiated arrays. When considering SNR values, the non irradiated arrays displayed a ratio of 12, while the irradiated ones gave a value of 100. Based on this evidence, it was unambiguously concluded that the non specific signal arising from thiolated oligonucleotide bound to the surface is negligible. Furthermore, it is possible

to assume that the described procedure (Section 2.4.1) allows the selective bonding of thiolated oligonucleotides on SU-8 BD surfaces, which implies potential utilities when spatial resolution is required.

Figure 2 confirms the effective light-mediated anchorage of thiolated probes to epoxy-coatings by the formation of a covalent bond and its quantification by fluorescence measurements. The irradiation time employed (10 min) sufficed to bind the thiolated oligonucleotides to the surface. A low non specific signal was observed from the immobilization negative control line (SYM41). In fact, when relating the oligonucleotide signal to the negative control (both printed at 5 μM), a ratio of 10 was obtained. Regular round-shaped spots were observed on every line.

FIGURE 2

As previously mentioned, the covalent bond formation between the SH-oligonucleotides and the epoxy groups located on the surface of the SU-8 coating was light-mediated by a thiol-epoxy chemistry process. Hence the influence of irradiation time was studied on the SH-oligonucleotide-Cy5 (SYM44) attachment to the modified BD surface. The results reveal that the non specific signal drastically increased with irradiation time; with SYM44/SYM41 (both printed at 5 μM), neat signal values of 10, 6 and 2 (for 5, 10 and 15 min, respectively) were achieved. Thus an irradiation time of 5 min was assumed convenient and was used from that time onward.

3.4. Optimal hybridization conditions for the 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 (SYM43/SYM5 system). Figure 3 depicts the fluorescence from a hybridization of 20 μM of probe SYM43 and 0.5 (or 0.1 μM) of tracer SYM5. Both concentrations were previously adjusted to obtain the strongest non saturated signal on the BD-player. The optimal system conditions were obtained at 30 min., 37ºC and 0.234 M ionic strength.

Figure 3A shows the fluorescence signal measured on hybridized arrays after different incubation times ranging from 15 to 90 min. In this study, temperature and ionic strength were kept constant at 37ºC and 0.234 M, respectively. When hybridization was assayed for 15 min, the resultant fluorescent 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 irrespectively of incubation time, indicating that every accessible probe coated on the surface is already bound to a DNA target. So carrying out the hybridization step for 30 min was established.

FIGURE 3

Figure 3B retrieves the fluorescence hybridization curves for different temperatures ranging from 15 to 65ºC. The data shown correspond to a hybridization achieved with probe and target concentrations of 20 and 0.1 μM, respectively, and an incubation period lasting 30 min, and an optimum hybridization value was achieved at 37ºC. This value is coherent with the hybridization tendencies described in the literature, which depends on the bases 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 Figure 3C. 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 37ºC. As previously described for the hybridization temperature, a curve with a maximum fluorescence value was obtained. Here, 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 strands repulsion and hybridization events.

3.5. DNA hybridization determined by enzymatic development

Having optimized the hybridization process, a step forward was challenged in order to employ the BD-player for the assay read-out and to work out the achievable limit of detection when DNA hybridization was enzymatically developed on SU-8 spin-coated BD surfaces. For this purpose, the interaction of a thiolated DNA probe to its complementary DIG-tagged strand (PAT16/PAT3) was studied using a-DIG/HRP as a tracer and TMB as the developing solution [8]. Note that both PAT16 and SYM43 have a 36 similar sequence of bases (18T, 6G, 5A and 7C for PAT6 and 20T, 4G, 5A and 7C for SYM43) and a molecular weight close to 11.5 kDa. By assuming that both oligonucleotides would behave the same way, SYM43 hybridizing optimal conditions were applied to the PAT16 assays (Section 3.4). The signal generated by the enzymatic reaction on the BD/SU-8 surfaces was recorded with the BD-player device prototype (Section 2.5).

The images corresponding to the assayed arrays (Figure S4, ESI) allow 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 is 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" is 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 [34]. For the SU-8 substance, a ratio of 3 epoxy units/1.1 nm^2 surface was calculated after the modelization, using licensed Chem3D ultra software, of an oligomer constituted by three repeated units. The relationship between both surface occupancies resulted in 68 epoxy groups covering the same surface as one SA molecule. Thus, SU-8-modified surfaces would promote higher hybridization yields when comparing covalently bound SH-oligonucleotides to physically adsorbed biotinylated oligonucleotide/SA systems.

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 10000 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 (the SYM50 mean value plus 3 times its standard deviation). These results confirm that hybridization can 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 PAT16 probe with 0.1 nM of the PAT3 target under the previously optimized conditions. Intradisk and interdisk reproducibility of 6% and 14% was achieved, respectively.

3.6. Application to the detection of Salmonella typhimurium DNA

Finally, the utility of the developed surfaces as microarray platforms for real applications was studied. For this purpose, the PCR-amplified DNA products form 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 PAT16 probes on the SU-8 film deposited on a BD surface was complementary to the central region of a 152-bases amplicon specific to detect an innocuous serotype of *S. typhimorium*. Next 200 nM PAT16, as a probe, and SYM41 and SYM50, 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 (Section 2.4.3), deoxiribonucleotides 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 Figure 4.

FIGURE 4

A range from 1/20 to 1/200 of amplicon dilution factors was studied for the hybridization assays. The arrayed spots containing a dilution as low as 1/80, i.e., 20 pM of the PAT16 complementary probes to the amplicon, showed a BD-player signal after a-DIG/HRP tracer development with TMB. Other diluted samples displayed no confident values. Note that a sensitivity of 1 pM was achieved after the hybridization between PAT16 and its full complementary target PAT3. However, the use of amplicon as a target lowered sensitivity to 20 pM. Such behavior is consistent with a more marked hindrance and disturbances occurring when longer DNA strands are implicated (152 bases in the amplicon *versus* 26 bases in the PAT3 oligonucleotide). Non complementary 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 reveal an improvement in the limit of detection to any of the referred examples; i.e., the hybridization carried out on SU-8 coated-glass surfaces [35] and on DVDs [8], 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.

4. 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 help 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 [36] 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 detector of the assayed arrays 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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Tables

Table 1. Nucleotide Sequence of Probes and Targets Used.

Table 1. Nucleotide Sequence of Probes and Targets Used.

Name	Sequence (5' to 3')	5'-end	3'-end	Role
PAT3	DIG-AGGGTCGTACACCGGCTGTAATCAAA	Digoxigenin	-	Target
SYM5	Cy5-AATGCTAGCTGG TCAATCGGG	Cy5	-	Target
PAT16	SH-T_{12}-GATTACAGCCGGTGTACGACCCT	Thiol	-	Probe
SYM41	CCCGATTGATTAGCTAGCATT-Cy5	-	Cy5	Control
SYM43	SH-T_{15}-CCCGATTGACCAGCTAGCATT	Thiol	-	Probe
SYM44	Cy5-TTACGATCGACCAGTTAGCCC-T_{15}-SH	Cy5	Thiol	Probe/target
SYM50	SH-T_{15}-CGCCGATAACTCTGTCTCTGTA	Thiol	-	Control

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

Figures

Figure 1

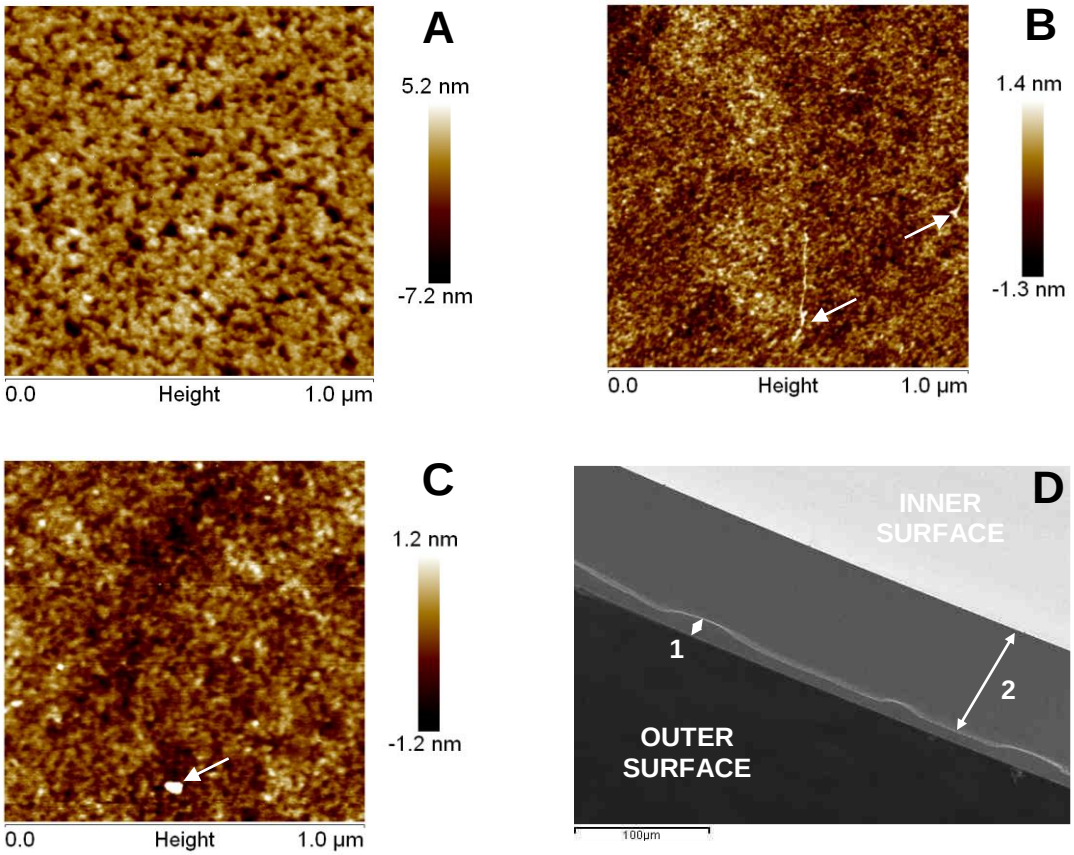

Figure 1. AFM images corresponding to bare BD surface (A), SU-8 spin-coated BD surface (B) and DNA hybridized on SU-8 BD modified surface (C). Analysis developed on a Veeco Multimode AFM microscope. (D) SEM image corresponding to a cross-section of the BD upper layer film (labeled as 2) spin-coated with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

Figure 2

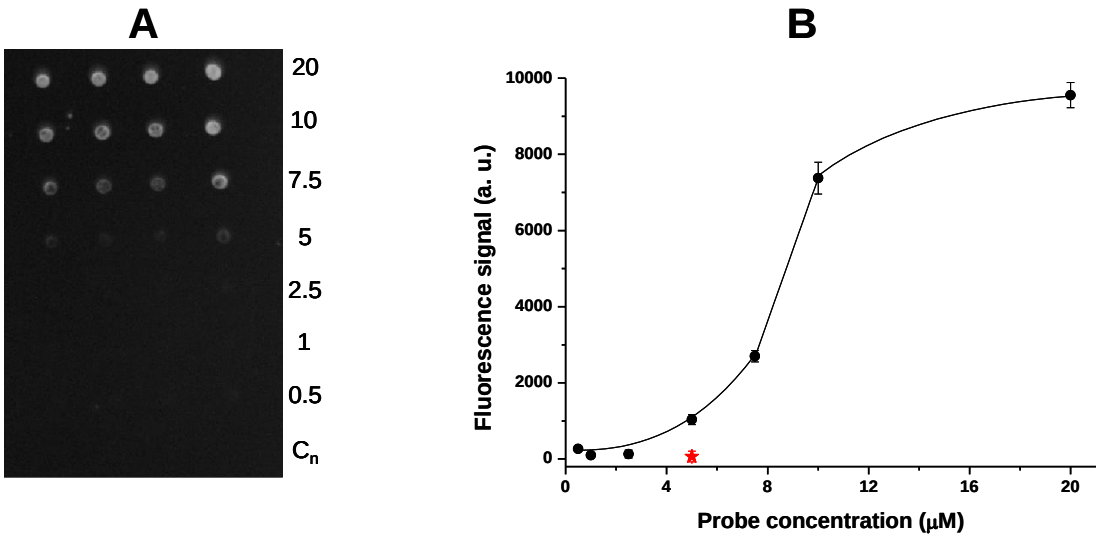

Figure 2. A) Fluorescence image corresponding to the assayed array (values refer to oligonucleotides concentration in μM units). C_n, negative control (SYM41) printed at 5 μM. B) Fluorescence data as a function of oligonucleotide concentration associated to the assayed array. Unspecific attachment negative control value corresponds to SYM 41 5 μM (red star).

Figure 3

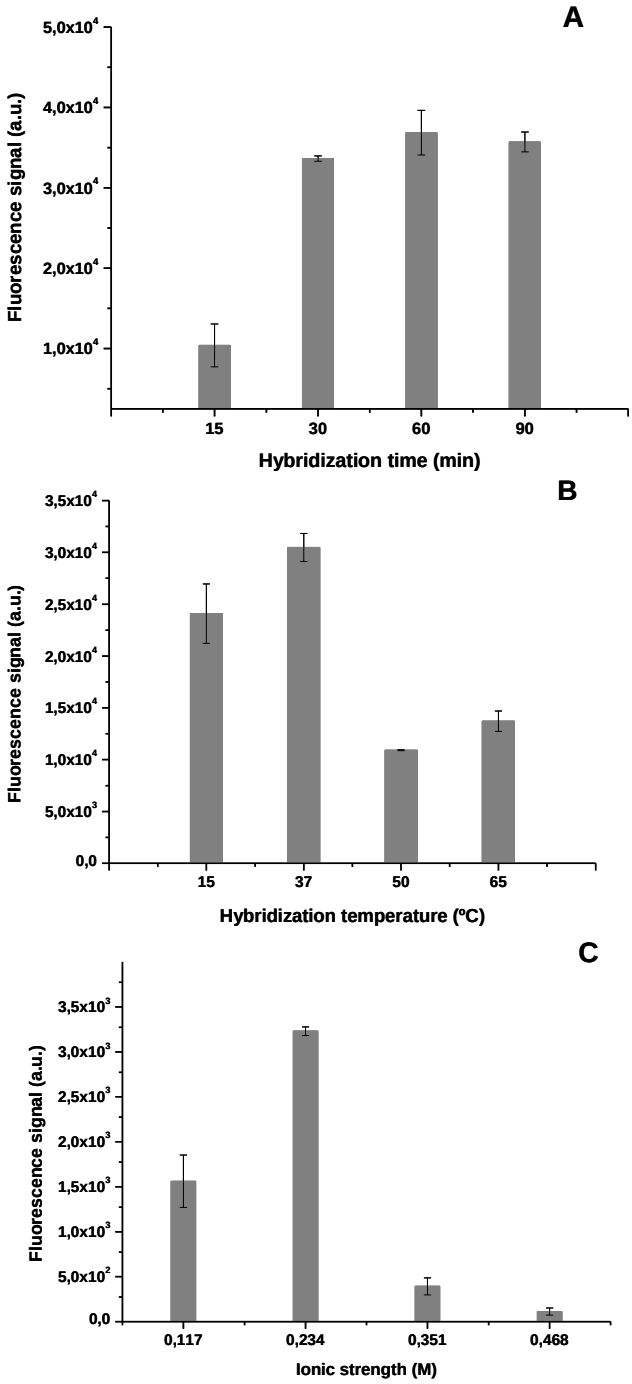

Figure 3. Optimization of the hybridization time (A): SYM43 probe and SYM5 target concentrations were 20 μM and 0.5 μM, respectively. Experiments carried out at 37 ºC and 0.234 M (ionic strength). Optimization of the hybridization temperature (B): SYM43 probe and SYM5 target concentrations were 20 μM and 0.1 μM, respectively. Experiments carried out for 30 min and 0.234 M (ionic strength). Optimization of the hybridization ionic strength (C): SYM43 probe and SYM5 target concentrations 20 μM and 0.1 μM, respectively. Experiments carried out at 37 ºC for 30 min.

Figure 4

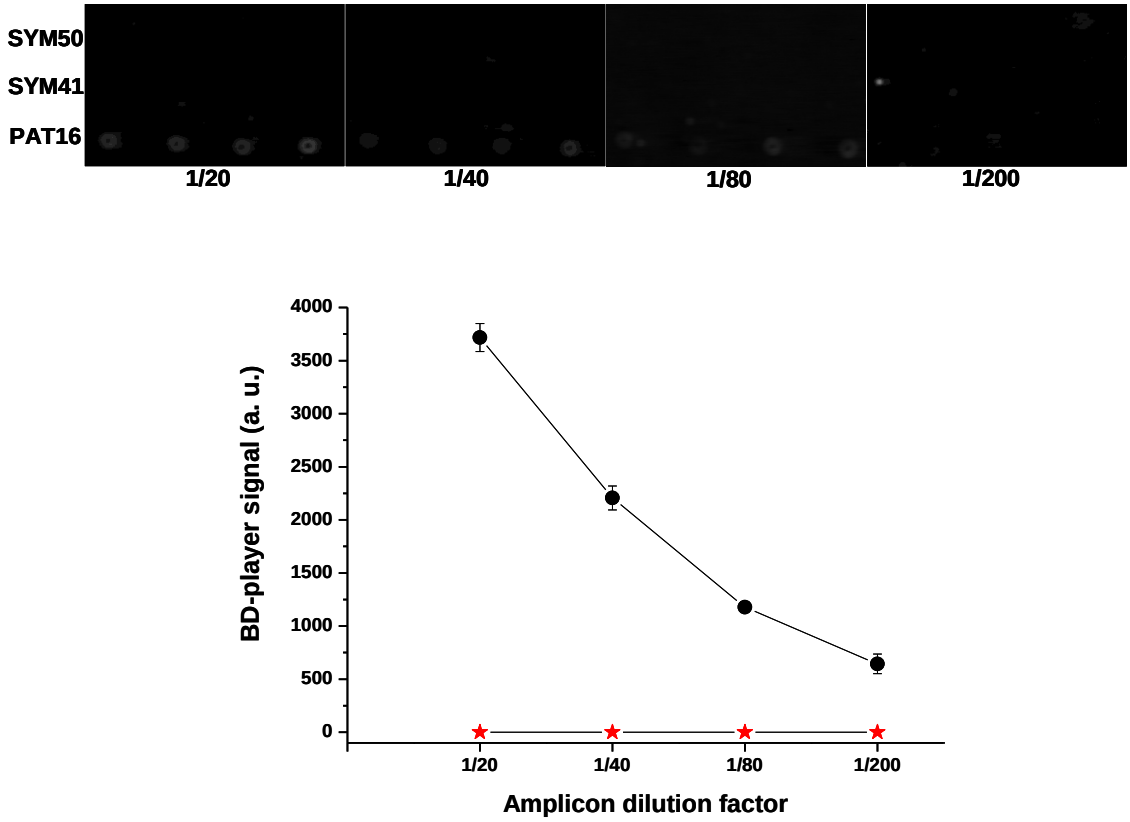

Figure 4. Top images show the sequence of assayed areas as captured by the BD-player device (type of probe appears on the left and values beneath each image refer to target amplicon dilutions). Graph reflects the BD-player signal corresponding to the hybridization of PAT16 probe with *S. typhimurium* PCR product (37 ºC, 30 min 0.234 M in SSC 2x). Hybridization negative control coated at 200 nM (star symbol).