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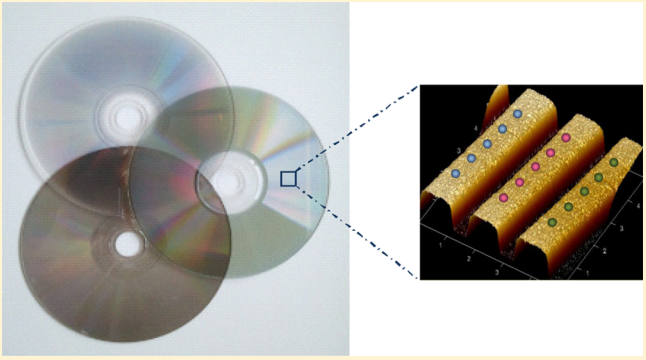
# Gold, Carbon, and Aluminum Low-Reflectivity Compact Discs as Microassaying Platforms

Eva M. Brun, Rosa Puchades, and Ángel Maquieira*

Centro de Reconocimiento Molecular y Desarrollo Tecnológico, Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain

Supporting Information

**ABSTRACT:** Compact disc (CD) surface modification with different reflective materials offers new working perspectives as bioanalytical platforms. The potential of gold, carbon, and aluminum low-reflectivity compact discs as a substrate for microassaying is presented. CD polycarbonate bases were coated with these reflecting materials, maintaining optical properties. Probes immobilization onto reflective layers was studied by both passive adsorption and covalent linking. Different chemical modifications were made on substrates to provide functional groups capable of anchoring probes with primary amines covalently. Thus, self-assembled monolayers were performed by chemisorption of 11-mercaptoundecanoic acid on gold surfaces, and silanization with *N*-(trimethoxysilylpropyl)ethylenediamine triacetic acid on aluminum to provide a carboxylic acid functional group. Carbon oxidation with oxygen plasma afforded similar functionalization on the discs coated with this material. Performance of the studied materials as reflective layers was evaluated, and as proof of concept, a microimmunoassay for a neurotoxic compound was studied on the three surfaces. The results show the possibility of doing assays on this new supports with good analytical performances while maintaining discs' optical and mechanical properties to be read by a CD player.



Electrooptical devices derived from mass-consumed electronics, such as compact discs (CDs), cellular phones, TV and computer screens, etc., have a high potential to be used as a basis for developing biosensing systems. Among them, CD technology offers enormous possibilities for the high-throughput analysis of specific biointeractions.[1] The advantages of audio/video CD as supports[2−6] include their large surface to spot thousands of capture microarrayed probes, low fluorescent background, high-quality low-cost materials, quadratic, circular, and spiral indexing, easy handling and manufacture, scalability, and the possibility of combining the detection and recording of the analytical results developed on the same disc. Additionally, the use of CD audio/video players as detectors makes their application as point-of-care and low-cost analytical systems possible, which is especially interesting for biomedical diagnostics.[3,6−11]

Conventional CDs are made from a 1.2 mm thick disc of polycarbonate (PC) with a spiral track on the top, coated with a reflective layer and protected by a lacquer resin with outstanding physical properties (Figure 1).[3] A CD is read by focusing a 780 nm wavelength laser through the bottom of the PC layer and registering the light reflected. Sensing on the CD polycarbonate face has been exploited, but we still have far to go. One possibility is to take advantage of the CD reflective metallic layer, onto which analytical assays can be developed combined with a transmission reading mode instead of the standard reflection one. There are many metals that can be potentially applied to make CDs, of which aluminum, nickel, silver, and gold are the most used. Other metals, such as chromium, titanium, copper, zinc, tantalum, and materials like graphite and silicon, are also utilized to manufacture CDs on a commercial scale.

The prospective of this approach is huge. Besides the valuable full technology of CDs, discs, and readers, the use of different metals as surfaces onto print microarrays to develop analytical assays is a challenge, and important issues should be resolved. The first refers mainly to the metal to be selected since each element or allow has particular optical and mechanical properties, followed by establishing the metal anchoring mode to the polycarbonate CD substrate, the optimal thickness of the reflective layer, the finishing (roughness, hydrophobicity, etc.), the probe's (mainly proteins and nucleic acids) immobilization strategies (adsorption, covalent and affinity attachment, or diffusion), and the reagents applied to develop biosensing assays.

The literature shows that the deposition of different reflecting materials and probes anchoring on the surface of CDs for analytical applications is a poorly studied area, except for a few research works which delved into the surface plasmon

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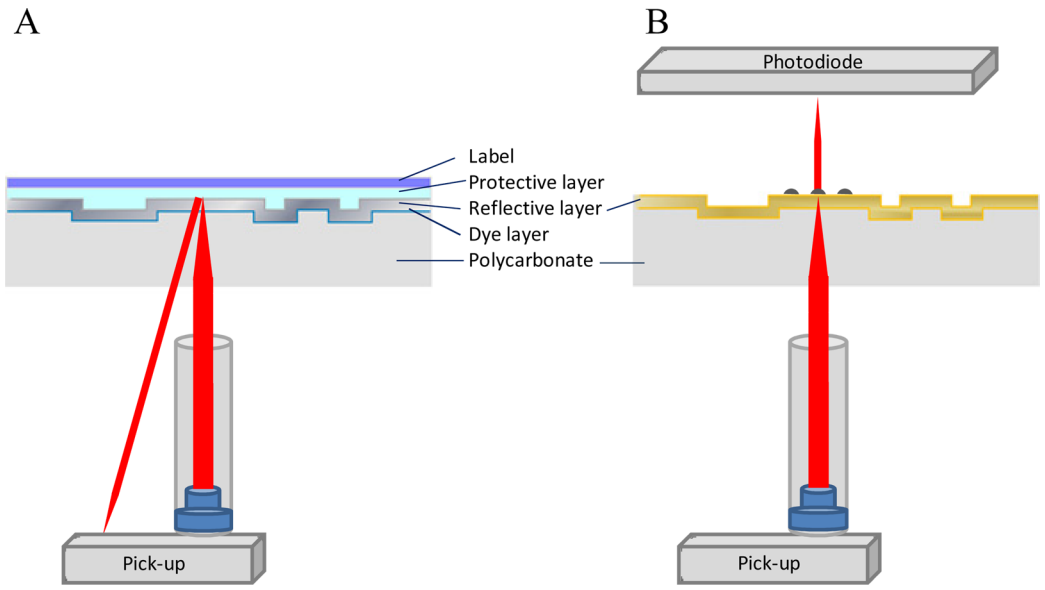


**Figure 1.** Scheme of the reading by (A) the reflection mode in conventional CD-R and (B) the transmission mode in L-CD.

resonance (SPR) phenomenon on CDs.[12,13] Other works report interferometric biosensors by the deposition of radial spokes made of gold ridges on a high-reflectance substrate, e.g., a silicon wafer.[14,15] Recordable gold CDs have been utilized to prepare alkanethiolate self-assembled monolayers,[16−18] electrodes, and microchips for electrochemistry studies,[19,20] or to produce material microstructures.[21] All the above-mentioned approaches damage the disc structure, track, and surface, which are no longer readable by a standard drive.[22]

In this work, for the first time the bare reflective layer of low-reflectivity compact discs (L-CDs) was used directly as an analytical platform, in combination with the disc drive as an optical readout device, using transmission phenomena for reading purposes (Figure 1). The use of transmission mode disc reading is scarce, and analytical platforms utilized are commercial gold L-CDs.[3,6,7,11] Transmitted light is detected by a simple planar photodiode incorporated into the CD drive[3] which converts it into an electrical signal. In the absence of analytical responses, the laser light is transmitted through the surface of the L-CDs and is detected as a background signal. Conversely for a positive signal, the optical properties of the disc surface change, leading to a variation in the light intensity detected by the photodiode, which further relates to the analyte concentration. Gold L-CDs have opened up analytical applications for CDs by working on the top side of discs, where the gold layer has been lacquered with a film of polycarbonate,[3] poly(methyl methacrylate),[7] or polystyrene.[11]

Thus, except for gold covered with a polymer, the reflective layer of the CD has not been investigated as probe anchoring surfaces. In this work, we develop new transparent CDs by the deposition of different reflecting materials onto compact disc polycarbonate bases. This goal is achieved by sputtering and flash evaporation techniques that do not harm the CD optical properties being recognized by a conventional CD player. A compromise must be reached: the coating layer should partially reflect the CD player laser and, at the same time, transmit an enough intense beam to develop the analytical signal.

The use of L-CDs allows us to use the laser from the standard CD drive ($\lambda$ 780 nm) as the only light source to generate the analytical signal, while gradually tracking across the CD surface to do chemical assays on the top side of discs.[3] Moreover, the disc transmission reading mode offers a major benefit in terms of spatial resolution because the laser light incident on the polycarbonate down side is refracted at a higher angle onto the surface; thus, the original incident spot diameter of around 700 $\mu$m focuses down to 1.7 $\mu$m on the reflective layer, thus achieving a higher optical resolution.[3]

In order to obtain compatible high-throughput analytical platforms with CD technology, both the covalent coupling and passive adsorption of bioreceptors on the performed reflecting disc layers have been studied. A variety of reflectant materials has been tested, as they offer diverse optical and physicochemical properties: reflectivity, transmission, roughness, hydrophilia, covalent functionalization vias, etc., which can improve immobilization yields, reduce nonspecific binding, provide new functionalities, and increase the reading resolution. In this study, low-reflectivity gold-, carbon-, or aluminum-coated CD bases as supports are assayed (Figure 2). Moreover, an immunoassay for the organophosphorus pesticide chlorpyrifos is assessed as proof of concept. The goal of this work is to make full advantage of CDs' potential reflective materials to accomplish new functionalities and better analytical performances.

## METHODS

**Chemicals.** Sodium phosphate buffers (PBS, 8 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4; PBS-T, PBS containing 0.05% (v/v) Tween 20) were filtered through a 0.22 $\mu$m pore size nitrocellulose membranes from Whatman GmbH (Dassel, Germany) before use. Antichlorpyrifos polyclonal rabbit antibodies (BSA-C2-II) and coating conjugate (OVA-triclopyr) were previously home-obtained and characterized by enzyme-linked immunosorbent assay (ELISA).[23] Chlorpyrifos standard was purchased from Dr. Ehrenstorfer (Augsburg, Germany). *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), Tween 20, 11-mercaptoundecanoic acid (11-MUA), 5 nm colloidal gold-labeled goat antirabbit immunoglobulin (GAR−Au), ovalbumin (OVA), silver enhancer solutions, tetramethylbenzidine liquid substrate


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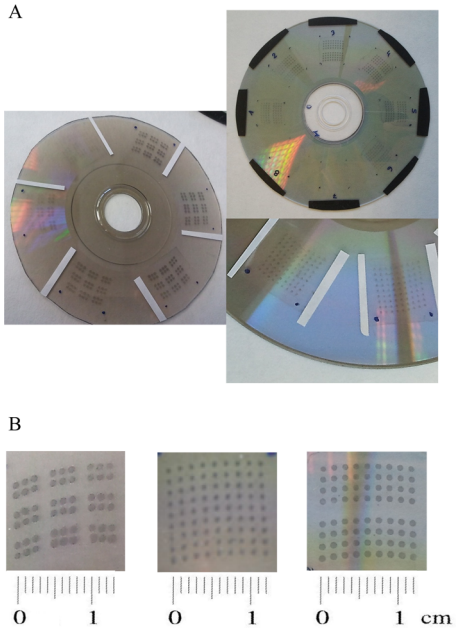


**Figure 2.** (A) Photographs of the developed arrays on L-CDs. Clockwise from left: carbon, gold, and aluminum. The black and white segments on the CDs are trigger footprints, which delimitate the analytical areas to be captured by the CD driver. (B) Left to right: detail photographs of the arrays on carbon, aluminum, and gold discs.

(TMB), Tween 20, and bovine blood hemoglobin (Hb) were supplied by Sigma-Aldrich (Madrid, Spain). *N*-(Trimethoxysilylpropyl)ethylenediamine triacetic acid, trisodium salt, 45% in water, was purchased from Gelest, Inc., (Morrisville, PA). Note: all the chemicals should be handled by following the corresponding material safety data sheets.

**Instrumentation.** Sessile drop contact angle measurements were performed with a Data Physics contact angle system OCA (Filderstadt, Germany) by applying a 10 $\mu$L drop of Milli-Q water (18 MΩ) to a freshly prepared surface. The values reported herein are the averages of five separate drops per surface. All the measurements were taken at room temperature.

Spot size was determined with a Leica MZAPO stereoscopic microscope (Leica Microsystems GmbH, Wetzlar, Germany) as the mean of six spot diameters.

Cyclic voltammograms were recorded using an Autolab model PGSTAT 100 (Metrohm, Switzerland) potentiostat/galvanostat, in an aqueous electrolyte containing 5 mM $KNO_3$ and 5 mM $K_4Fe(CN)_6$. A saturated calomel electrode (SCE) was used as the reference electrode in electrochemical experiments, and potential values are reported in relation to the SCE.

Atomic force microscopy (AFM) images were recorded by a NanoScope III from Bruker (Santa Barbara, CA) operating in the tapping mode in air. Si-cantilevers from Bruker, resonance frequency of 75 kHz in soft-tapping conditions, were used. The ratio between the set-point amplitude and the free amplitude of cantilever vibration was always maintained above 0.8.

X-ray photoelectron spectroscopy (XPS) was performed with a SPECS SAGE 150 photoelectron spectrometer (Specs, Berlin, Germany).

For the automatic application of spots in microarray format, a noncontact printing device was used (AD 1500 BioDot, Irvine, CA, U.S.A.). The reproducibility of the delivered volume (20 nL) was ensured by the steady-state pressure (SSP) inside the dispensing channel. In addition, working temperature and relative humidity were controlled (25 °C and 90%, respectively).

**Analytical Platforms.** Compact disc polycarbonate bases (without metallic and protective layers) provided by U-Tech Media Corporation (Tau-Yuan Shien, China) were used as platforms. Prior to deposition, discs were cleaned with deionized water and dried by mild centrifugation.

Gold CDs were produced by sputtering gold deposition with a cool sputter coater model, Leica EM SCD005 (Leica Microsystems GmbH, Wetzlar, Germany). Supporting Information Figure S1A shows an AFM image of the top gold layer of an L-CD and the corresponding cross-sectional profile.

Golden discs were functionalized by self-assembled monolayer (SAM) formation. First the disc was conditioned by gentle ethanol washing and dried by centrifugation (840 rpm). Carboxylic acid-terminated monolayers were prepared by immersion of the gold substrate in a 25 mM ethanolic solution of 11-MUA for 24 h. Then, the disc was washed with ethanol, rinsed with water, and dried by centrifugation.

Carbon deposition was performed in a Leica EM SCD005 carbon thread evaporation device (Leica Microsystems GmbH, Wetzlar, Germany) under prevacuum conditions (60 Pa) by flash evaporation process (sublimation of a carbon thread). Due to the small dimensions of the vacuum chamber, 8 cm diameter CDs were used. An AFM image of a carbon CD is shown in Supporting Information Figure S1B.

Carbon surface oxidation was carried out by oxygen plasma treatment. Discs were introduced inside a microwave plasma reactor PVA Tepla 200 Plasma System (Feldkirchen, Germany) operating at 2.45 GHz, with a flow rate of 75 sccm and at continuous 100 W power for 30 s. The oxygen pressure inside the reactor was 100 Pa. Discs were stored under vacuum and dry conditions until use.

Aluminum deposition was performed by sputtering using a K675X sputter coater (Emitech, Kent, U.K.). Supporting Information Figure S1C illustrates the AFM image of an aluminum CD. The aluminum surface was cleaned and activated by nitric acid treatment. Then, it was silanized with *N*-(trimethoxysilylpropyl)-ethylenediamine triacetic acid trisodium salt by immersing the surface into a solution of 1% silane (v/v) in citrate buffer, pH 5.5, for 10 min and cured at 110 °C for 30 min.

The chemical composition of the functionalized layers was determined by XPS and the thickness of the deposited layers by a scratch technique and AFM.

**Covalent Immobilization of Proteins onto Carboxylic Acid Modified L-CDs.** Disc surface activation was carried out by dispensing 1 mL of 25 mM NHS and 125 mM EDC in Milli-Q water. The disc was covered with a polycarbonate dummy surface (Cóndor CD, Madrid, Spain) for 60 min at room temperature, rinsed with deionized water, and dried.

Immediately, a solution of protein (20 mg/L OVA-triclopyr in Milli-Q water) was microarrayed on the L-CDs, eight arrays of 9 × 9 (6 arrays of 9 × 6 for the carbon discs). The coupling reaction was carried out for 16 h at 4 °C under saturated humidity conditions. Finally, discs were thoroughly rinsed with PBS-T and water and dried by mild centrifugation.

**Passive Immobilization of Proteins onto L-CDs.** OVA-triclopyr conjugate (20 mg/L in Milli-Q water) was directly microarrayed on the L-CDs, eight arrays of 9 × 9 (6 arrays of 9 × 6 for carbon discs). Adsorption was performed at 4 °C for 16


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h under saturated humidity conditions, and surfaces were washed and dried as described before.

**Indirect Competitive Microimmunoassay Protocol.** After covalent or passive immobilization of the coating conjugate, a blocking step was carried out to prevent nonspecific adsorption or to deactivate residual NHS ester groups. Thus, a hemoglobin solution (1% in PBS) was dispensed onto the disc, covered with a PC dummy surface for 60 min, then washed with PBS-T and deionized water, to be finally dried by mild centrifugation.

Subsequently, the arrays printed on the disc were coated with 100 $\mu$L of the specific antibody solution (BSA-C2-II diluted 1:1000 in PBS-T) containing different concentrations of the analyte. After 10 min, the disc was washed, and 100 $\mu$L of secondary antibody (GAR−Au, 1:25 in PBS-T) was dispensed onto each array. After 30 min at room temperature, the disc was washed as described before. In order to display the immunoreaction, arrays were incubated with 1 mL of 1:1 (v/v) silver enhancer solution, and the reaction was stopped after 6 min by washing the disc with water. After drying, the results were read in a CD drive. A scheme of the microimmunoassay is shown in Figure 3.

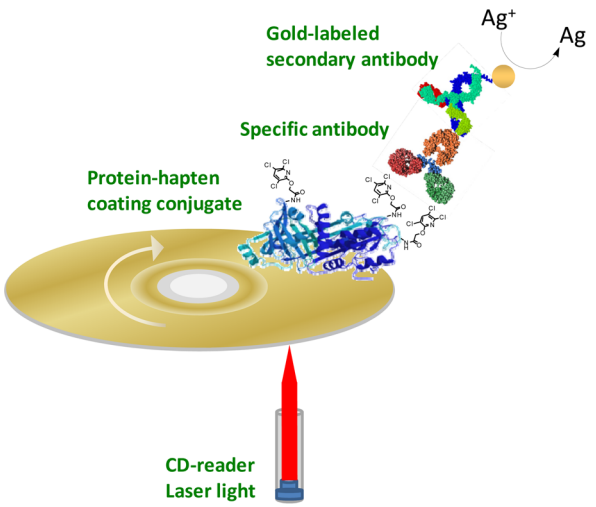


**Figure 3.** Schematic representation of the microimmunoassay carried out on compact discs.

**Detection System.** A commercial optical disc drive (Premium, Plextor America, Fremont, CA) was used as detector by incorporating a photodiode.[3] The drive has an optical system with a laser ($\lambda$ 780 nm) to read standard CDs and uses the servo focus/tracking system to center and focus the beam on the spiral data track of the entire disc surface. The transmitted light through the disc is transformed into an analog electrical signal by a photodiode [SLSD-71N6 from Silonex (Montreal, Canada), 25.4 mm long and 5.04 mm in width, with a spectral range between 400 and 1100 nm, maximum spectral sensitivity of 0.55 A/W at 940 nm]. The electrical signal generated by the photodiode is digitalized by a 16-bit data acquisition board DT9832A-02-OEM from Data Translation Inc. (Menlo Park, CA, U.S.A.), stored in memory, and then deconvoluted into an image. The captured data are transferred to the computer through a universal serial bus 2.0 interface for its quantification.

The reading of assay results was performed using a custom software written in Visual C+ (BioDisk) which simulates the writing process of a 700 MB size file. This software also controls the data acquisition board, selecting the sampling frequency, size file, and writing the resulting data to the computer hard disc in an uncompressed binary format.

Reading areas of the discs were selected by trigger footprints on the outer rim of the CD, or between areas (Figure 2A), and detected by a reflective photosensor EE-SY125 from Omron (Schaumburg, IL, U.S.A.), which is integrated onto the custom-built electronic board. Because the unmarked perimeter presents higher reflectivity, the photosensor detects the marked areas providing a trigger signal to the data acquisition board in order to start capturing data exclusively from those zones. This leads to reduced data files, saving memory in the computer. The data captured from each detection area were represented within a sector that is formed by a set of arcs centered over a radial direction, starting from the most inner and moving toward the outer radius, so an image in a 16-bit gray-scale code was obtained for every sample area.

**Imaging and Data Analysis.** The Biodisk software allows for the image to be transferred onto a 16-bit tif format or bitmap. Due to the spatial difference between samples taken horizontally and vertically, a graphical adjustment was made to display a proportional $X-Y$ image with Photoshop 7.0 from Adobe Systems Inc. (San Jose, CA, U.S.A.). The quantification was made by GenePix software from Axon Instruments (Union City, CA, U.S.A.). Absolute signal intensity of each spot was calculated by subtracting the corresponding background. Inhibition curves were mathematically analyzed by fitting experimental results to a sigmoidal four-parameter logistic equation. All the software implemented runs on a Windows-based personal computer.

## RESULTS AND DISCUSSION

**Reflective Films Deposition and Thickness Optimization.** Since the purpose of this study was to assess compatible assays with the CD player, the optimal composition and thickness for each film material was the minimum that allowed the reader to recognize the disc, thus providing the best laser transmission and surface finishing (roughness and hydrophobicity).

In order to determine the optimal gold layer, CD polycarbonate bases were gold-metallized for different times: 20, 30, 40, 50, and 60 s, yielding gold film thicknesses of 10.2 ± 0.5, 14.3 ± 0.4, 18.9 ± 0.5, 24.3 ± 0.6, and 28.1 ± 0.5 nm, respectively (AFM record, Supporting Information Figure S2). If sputtering lasted less than 20 s, the gold layer thickness was not reproducible. By working in this mode, good gold adhesion was achieved, which is key to produce a stable analytical substrate.

The influence of gold film on the analytical signal was studied. The increase in metal thickness lowered the transmission of the CD player laser through the disc, and as a result, a decreasing signal-to-noise ratio (S/N) was recorded (Supporting Information Figure S3). A S/N value statistically higher than 20 for blank samples (with no analyte) is considered acceptable because it corresponds to a S/N of 10 at $IC_{50}$, according to the IUPAC definition for the limit of quantification (LOQ). Only the disc with a 10 nm gold thickness accomplished an acceptable S/N ratio. Thus, it was chosen as being optimal to obtain a suitable absolute biosensing signal.

For carbon coating, the flash evaporation technique was chosen because the obtained films are fine grain, with uniform


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and reproducible film thickness. The major drawback of this technique is the asymmetry of deposition. However, it was resolved by rotating the disc 180° between coatings; therefore, an even number of coatings should be done to obtain the highest homogeneity. Another consideration relates to the variation of coating from the center to the outer edge. This effect is directly proportional to the proximity between the surface and the carbon filament. Minimal variation with good deposition efficiency was achieved by placing the disc 5 cm from the carbon source. Four depositions at this distance (mean thickness of 15 nm, measured by AFM) were required for the CD reader to recognize the disc. The radial homogeneity of the deposited layer was also examined by measuring thickness, which varied between 38.6 ± 0.6 nm in the center and 31.8 ± 0.8 nm on the outer edge. We observed that systematic coatings were highly reproducible and the carbon adhesion to the polycarbonate substrate was very good.

For aluminum CDs, different sputtering times (10, 15, 20, and 25 s) were tested, 20 s being the minimal to attain good coverage by affording a homogeneous film thickness of 16.7 ± 0.6 nm. A longer sputtering time led to poorer laser transmission. Good aluminum adhesion was also obtained.

Supporting Information Table S1 shows the layer thickness, reflectivity, and transmittance of the developed L-CDs. Reflectivity of around 25−30% was the minimum necessary to recognize discs by the CD reader. However, the best transmittance at 780 nm (Supporting Information Figure S4) was achieved by carbon discs, which implied a better S/N in the subsequent assays.

**Chemical Functionalization and Characterization of CD Platforms.** Surface functionalization is key to reach the necessary properties to immobilize probes by adsorption or covalently. Therefore, the obtained L-CDs were derivatized with a carboxylic acid function by following different strategies: a SAM of 11-MUA on gold CDs, a SAM of a silane−carboxylic acid on aluminum CDs, and the oxidation of carbon discs by oxygen plasma.

It has been reported that surface cleaning is a key step to form monolayers on gold. Different solutions, like piranha or diluted nitric acid, have been widely used for this purpose.[24] With gold L-CDs, these solutions damaged the metal layer due to its thinness, and a more gentle cleaning procedure is required, so discs were conditioned with ethanol.

As previously described,[25] 11-MUA SAMs did not affect disc transparency or gold color, although thicker films of about 50 nm are commonly used.[19,26]

Similarly, aluminum film was not affected by silane SAM formation and carbon remained intact after plasma treatment.

*Surface Characterization. Wettability.* Sessile contact angles were randomly determined on different parts of the original or modified CDs ($n$ = 6) with a 10 $\mu$L drop of pure water. Supporting Information Table S2 shows the contact angle values for the studied surfaces. In all cases, surface functionalization with carboxylic acid groups diminished the contact angle, indicating a change to a more hydrophilic surface.

Spots size is directly proportional to surface hydrophilia. Thus, the more hydrophilic the surface, the bigger the spot diameters obtained, ranging from 560 on gold to 1168 $\mu$m on oxidized carbon for a printing volume of 20 nL.

It was observed that freshly made carbon films were highly hydrophilic, with $\theta$ < 20°. However, they become more hydrophobic with aging at room temperature and reached a constant value ($\theta$ about 45°) after 5 days (Supporting Information Figure S5).

*Surface Composition.* Gold, carbon, and aluminum surfaces were characterized by XPS before and after functionalization (Supporting Information Figure S6).

The XPS spectrum of an 11-MUA SAM developed on a gold L-CD (Supporting Information Figure S6A) showed the presence of sulfur on the modified surface and an increase in the carbon and hydrogen percentages (Supporting Information Table S3A). These results correspond well to those previously reported.[27] In Supporting Information Figure S7, detailed spectra of the S2p and C1s peaks are shown. The maximum for the S2p peak at 162.5 eV is characteristic of sulfur bonded to a gold substrate,[28] and the strong attenuation indicates that sulfur is indeed at the bottom of the SAM. Regarding the C1s signal, a binding energy of 284.8 eV is characteristic of the alkane chain. Moreover, the shoulder at around 289 eV is attributed to carboxylic carbon. Thus, the XPS results are in agreement with the features one would expect for a well-defined SAM.

Supporting Information Figure S6B shows the XPS spectra from both a carbon L-CD and an oxidized carbon L-CD. For carbon discs, peaks C1s and O1s correspond to 90% and 10% of carbon and oxygen, respectively. For the oxidized carbon discs, these values became 84% (C) and 16% (O) (Supporting Information Table S3B).

The spectra of the C1s peaks for carbon discs are shown as an inset in Supporting Information Figure S6B, where the position of −C− groups is observed with a binding energy of 287.7 eV. In the spectrum corresponding to oxidized carbon, the presence of −C−O−, −C═O, and −O−C═O structures at 287, 288, and 289 eV, respectively, is shown as a shoulder.

The XPS spectra from an aluminum L-CD, before and after silanization, is illustrated in Supporting Information Figure S6C. The change in the composition of C, Al, Si, and O (Supporting Information Table S3C) led to the confirmation of surface functionalization.

*Surface Finish.* The morphology of gold, carbon, and aluminum surfaces was investigated by AFM before and after functionalization. Supporting Information Figure S8 shows the topographical characteristics of the surfaces.

Minor differences in the images of bare gold CD and after SAM formation are depicted in Supporting Information Figure S8A. This agrees with other works[27] reporting the difficulty of demonstrating the presence of well-ordered monolayer on a smooth surface by the AFM technique. However, the variation in surface roughness from $R_a$ = 1.66 nm to $R_a$ = 1.40 nm was significant.

Flatter surfaces were obtained by sputtering aluminum ($R_a$ = 0.93 nm) and carbon deposition ($R_a$ = 0.92 nm), which became smoother after functionalization ($R_a$ = 0.61 nm for silanized aluminum and $R_a$ = 0.64 for oxidized carbon). For aluminum, the image after SAM derivatization showed more differences than gold. Yet once again, the verification of the SAM based on an image is a difficult task.

On the other hand, the surface modification with a monolayer can be verified with an electrochemical technique such as cyclic voltammetry. Therefore, this technique was used to characterize the monolayer formed onto aluminum and gold CDs. For aluminum, every attempt made to carry out this characterization failed. First, potassium ferrocyanide was used to obtain the voltammogram, but its oxidation was not visible because of fast aluminum corrosion. Other substances like 7,7,8,8-tetracyanoquinodimethane and $Ru(bpy)_3Cl_2$ were also


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tried, but must be solved in organic solvents that are incompatible with the CD polycarbonate layer. Therefore, this approach was not feasible.

With gold, cyclic voltammograms on bare and SAM-covered Au substrates were successfully recorded (Supporting Information Figure S9). The voltammograms indicated a densely packed monolayer because the redox reaction was completely blocked in presence of the SAM.

**Biosensing with CD Platforms.** Probe immobilization on the surface-based analysis of biomolecules is a critical step. In this work, protein immobilization was studied on the three analytical platforms developed either passively, through hydrophobic or ionic interactions, or covalently, by attachment to activated surface groups. Moreover, the influence of the immobilization strategy on the analytical immunoassay performances was assessed.

For this study, the coating conjugate was printed onto activated or bare gold, carbon, and aluminum surfaces. After a blocking step, the subsequent reaction with an antichlorpyrifos antibody and a secondary gold-labeled goat antirabbit antibody allowed us to develop an immunoassay with optical detection, using tracers yielding a precipitate on disc (Figure 3).

*Covalent Attachment of Immunoreagents.* It has been widely reported[29] that covalent binding may result in better biomolecule activity, less nonspecific adsorption, and great stability. Covalent attachment is often necessary to bind molecules that adsorb with improper orientation and conformation or very weakly on surfaces. Therefore, we started studying the covalent attachment of biomolecules in all the developed platforms.

A general procedure involves the use of a carboxy reactive surface, by first converting carboxylic acid functions into *N*-hydroxysuccinimide esters. This is a common method that provides good activity and yield results,[30] and it allows its application in different materials using organosilane and thiol chemistries.

Following this strategy, the SAM-modified gold CD was the first to be studied. As the density of the immobilized protein conjugate can affect the maximum signal, different OVA-triclopyr concentrations, ranging from 1 to 40 mg/L, were microarrayed onto the NHS-activated surface of a SAM-modified gold L-CD using a 1/1000 sera dilution. As expected, the S/N increased with the conjugate concentration (Supporting Information Figure S10A). However, poor quality and reproducibility of spots, due to excess reagents, were observed at concentrations higher than 20 mg/L. Thus, this was chosen as the optimal coating conjugate concentration to assess the immunoassay.

PBS buffer media, normally utilized to prepare proteins spotting solutions, led to "doughnut-type" spots at the end of the assay. The mechanism of such pattern formation, known as a "coffee-ring effect", has been widely explained on the basis of evaporation-driven convection.[31] Other authors have demonstrated a diffusion of protein molecules accumulated at the air/water interface to the perimeter of the droplet.[32] In addition, conventional buffered solutions used for microarraying spotting often contain a visible amount of salt. While the droplet is drying, the salt reaches its critical concentration and suddenly crystallizes at a nucleation point, which leads to non-homogeneous spots.[33] According to this, the best S/N results and the most well-defined spots were accomplished using only water as solvent to microarray OVA-triclopyr.

These days, however, glycerol is added to printing media to protect against potential protein denaturing effects and control spot morphology.[34] Different glycerol/water ratios printing solutions were assayed (from 1:99 to 10:90), but low S/N ratios were obtained in all cases. Therefore, pure water was used to dilute the coating conjugates.

After protein−hapten immobilization, several blocking agents were tested to deactivate the remaining active sites. Among 1 M ethanolamine, 1% OVA, 1% gelatin, and 1% hemoglobin in 1× PBS, hemoglobin led to a lower background signal.

The effect of antibody concentration is also an important factor in immunoassays signal and sensitivity. Thus, three sera dilutions were tested (1/500, 1/1000, and 1/2000). A lower S/N ratio was obtained with the 1/2000 dilution, while 1/500 did not improve the results. Therefore, a sera dilution of 1/1000 was chosen to continue the assay development.

The next step required a labeled secondary antibody capable of producing a precipitate after its reaction with a substrate to optically detect the analytical signal. In previous works,[3] the use of HRP as label and TMB as substrate provided the best results in terms of S/N ratios. Yet in the case of gold L-CD, these reagents removed gold and produced holes instead of spots. Then, a Au-labeled secondary antibody and a silver enhancer were evaluated. Initially, silver does not appear to be the best reactive to be applied to a gold surface. However, the SAM and the blocking step provide a film on the surface, avoiding the reaction between the CD gold reflective layer and the silver reagent, leading to satisfactory results.

Finally, discs were read in the transmission mode by a CD driver. The formed silver precipitate absorbed the light emitted by the reader laser, and the analogical signal variation correlated with the analyte concentration (see panels in Figure 4A).

In addition, in order to verify the selectivity of the reaction, a negative control (20 mg/L of OVA as coating conjugate) was tested, but no signal was obtained in any case.

Moreover, the thiol concentration for SAM formation proved critical for gold L-CD (Supporting Information Figure S10B). The immunoassay results showed that the lowest signals were obtained at low thiol concentrations (1 and 10 mM), while at higher levels (50 mM) the S/N ratio worsened. Hence, 25 mM was established as the optimal 11-MUA concentration for SAM formation on gold.

The chlorpyrifos immunoassays by covalent attachment onto carboxylic acid modified aluminum and carbon were carried out with the above-mentioned optimized parameters (20 mg/L of OVA-triclopir, sera dilution 1/1000, 1% hemoglobin in 1× PBS as a blocking agent, water as a coating solvent, GAR−Au and a silver developer). Only the SAM-modified gold L-CD provided a successful reading and analysis of data. A high S/N was obtained (Figure 4A, dotted line), the sensitivity ($IC_{50}$ 1.79 $\mu$g/L) being similar to that obtained by passive adsorption onto a polycarbonate film assembled on the top side of the disc ($IC_{50}$ 1.81 $\mu$g/L)[3] and better than that obtained on the down side ($IC_{50}$ 2.51 $\mu$g/L).[3]

Reading oxidized carbon discs proved difficult because the high surface hydrophilia led to big, poorly defined spots (Supporting Information Figure S11). Consequently, errors accumulated during disc reading, which hindered whole disc reading.

Although reading was complete in aluminum discs, "doughnut-type" spots were obtained (Supporting Information Figure S11). Thus, the mathematical analysis of the data did not depict a calibration curve.


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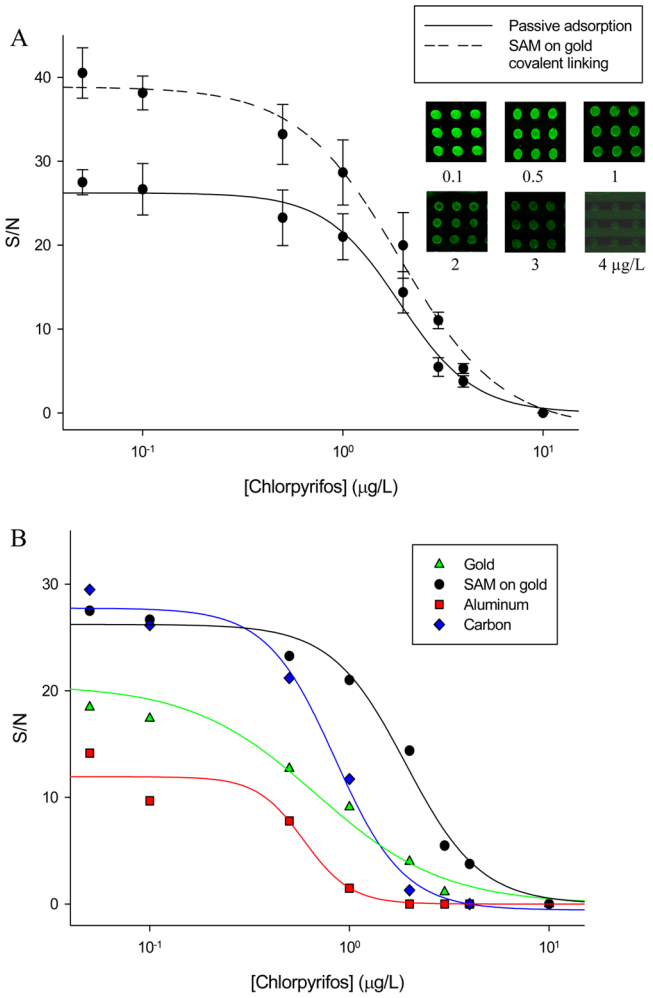


**Figure 4.** Calibration curves for chlorpyrifos ($n$ = 3) obtained by (A) covalent attachment (with representative images) and adsorption of immunoreagents onto a SAM-modified gold L-CD and (B) passive adsorption of immunoreagents onto the L-CD top layer of the studied materials. Error bars have been omitted to clarify the figure.

*Passive Adsorption of Immunoreagents.* Random protein physisorption on surfaces is the most common protein immobilization method thanks to its simplicity and swiftness. Nonetheless, orientation problems, irreproducibility, and protein conformational changes are common difficulties found.[35] As the covalent attachment of immunoreagents did not provide satisfactory results with all the studied L-CDs, a passive adsorption approach was tested on the three materials (gold, carbon, and aluminum). The previously optimized parameters were used to carry out the chlorpyrifos immunoassays.

With this approach, readable results were obtained on all the studied surfaces. Figure 4B shows the standard calibration curve for chlorpyrifos on the different platforms. Aluminum surfaces provided high sensitivity ($IC_{50}$ 0.59 $\mu$g/L) but a poor signal.

The best results were achieved by adsorption on carbon discs, with good S/N ratio (>25) and reproducibility and excellent sensitivity ($IC_{50}$ 0.85 $\mu$g/L).

In gold, both sensitivity and signal were good ($IC_{50}$ 0.69 $\mu$g/L). However, reproducibility was low, showing an interdisc coefficient variation of 29.8% (Table 1). A particularly useful approach for studying protein adsorption is the formation of SAMs as an intermediate layer. For this reason, adsorption onto the SAM-modified gold disc was tested. Supporting Information Figure S12 shows the AFM image before and after protein adsorption. In this case, reproducibility and the S/N were high, with a similar $IC_{50}$ (1.92 $\mu$g/L) to that obtained by covalent immobilization on the same gold surface ($IC_{50}$ 1.79 $\mu$g/L).

Therefore, excellent sensitivity was obtained on all the platforms, with carbon and SAM-modified gold being the surfaces showing better performance.

**Optical Resolution of the L-CD Upper and Down Sides.** One important benefit of working on the upper face of CDs is the greater optical resolution obtained when the disc is read with a CD player. On the down side of the disc, the focal diameter of the laser beam is 728 $\mu$m, so it was not possible to precisely resolve spots with smaller track pitches. Sensing on the top side of discs overcame this issue as beam crossing the polycarbonate substrate converges to emerge through the metal layer down to 1.7 $\mu$m. By working with narrow beams, higher optical resolution can be achieved, and the reproducibility of signals improves.

To illustrate this, different coating conjugate volumes (10, 20, 30, and 40 nL) were dispensed on both sides of a gold-coated L-CD. The upper side was previously modified with an activated SAM, whereas passive immobilization took place on the polycarbonate down side. The results are illustrated in Figure 5. All the spots were well-resolved by the CD player with no disruptions while reading. The optical resolution on the raw polycarbonate surface was clearly lower to that obtained on the top CD surface. Note that spot size was also lower on the down surface due to the higher hydrophobicity of polycarbonate as compared to the SAM-modified gold surface, on which the same volume of coating conjugate led to larger spots. Detection on the top side of standard CDs resulted in a good imaging resolution to readily sense as small as 13 $\mu$m diameter spots. Moreover, a major resolution led to a higher sensitivity (top side $IC_{50}$ 1.92 $\mu$g/L, down side $IC_{50}$ 2.56 $\mu$g/L). Similar results can be applied to all reflective layers on upper CD sides.

**Table 1. Analytical Performances of the Chlorpyrifos Microimmunoassay Developed on Different Disc Surfaces**

| surface | immobilization | sensitivity ($IC_{50}$) ($\mu$g/L) | limit of detection ($IC_{10}$) ($\mu$g/L) | dynamic range ($IC_{20}$–$IC_{80}$) ($\mu$g/L) | intradisc CV (%) | interdisc CV (%) |
|---|---|---|---|---|---|---|
| gold | adsorption | 0.69 | 0.14 | 0.26–1.94 | 15.5 | 29.8 |
| SAM-modified gold | adsorption | 1.92 | 0.75 | 1.08–3.59 | 8.3 | 11.9 |
| SAM-modified gold | covalent | 1.79 | 0.55 | 0.85–3.77 | 7.8 | 10.4 |
| carbon | adsorption | 0.85 | 0.35 | 0.49–1.41 | 12.8 | 14.0 |
| aluminum | adsorption | 0.59 | 0.33 | 0.41–0.88 | 16.6 | 18.3 |


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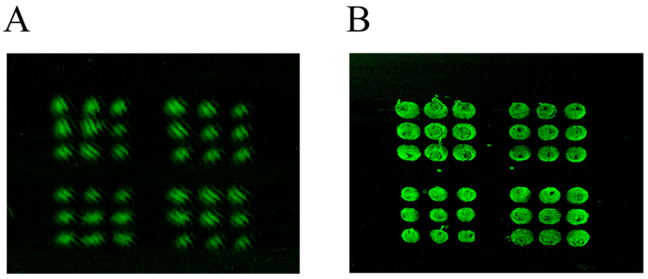


**Figure 5.** Optical resolution of the assays carried out (B) on the upper side of a SAM-modified gold surface and (A) on the polycarbonate down side. In both cases, the coating conjugate was immobilized by passive adsorption.

## CONCLUSIONS

Low-reflectivity compact discs, developed by coating raw polycarbonate CDs with gold, carbon, or aluminum, were functionalized by the self-assembling of a carboxylic-ended alkanethiol on the gold CDs surface, silanization of aluminum oxide with an acid silane, and oxidation of carbon surface by oxygen plasma. The CDs' modification preserved their physical and mechanical properties and allowed their readings on a standard CD reader/writer by incorporating a photodiode as detector. Surfaces characterization has shown lower contact angles and roughness for carbon and aluminum discs than gold CDs.

As proof of concept, an indirect competitive immunoassay has been assessed for the determination of the organophosphate pesticide chlorpyrifos on all surfaces, using different immobilization strategies. Results show that the achieved sensitivity was similar or better than that obtained by passive adsorption of the same immunoreagents on a polycarbonate film assembled on the upper disc side. A higher S/N ratio with good reproducibility was obtained by covalent binding onto a SAM-modified gold CD, although the poorest sensitivity obtained was shown on this surface. By comparing all the surfaces and immobilization strategies used, passive adsorption onto the carbon CD provided the best results in terms of sensitivity ($IC_{50}$ 0.85 $\mu$g/L, limit of detection $IC_{10}$ 0.35 $\mu$g/L), with a good signal-to-noise ratio (S/N 27 ± 1).

These results show that it is possible to develop new functionalities on CDs by coating them with very different reflecting materials (metallic or not) to provide new analytical platforms that are compatible with CD reading. This approach opens up the way for microarray-based assay development on a whole range of metals, oxides, composites, and biomaterials, which are potentially used in CD manufacture technology, with the optical resolution benefits of working on the top side of CDs. It is also possible to change the texture and physical properties of new surfaces, which is a very promising prospect. These approaches can be applied to other immobilization probes, such as nucleic acids, carbohydrates, and fatty acids. Furthermore, different functionalization reagents and chemistries, yielding amine, thiol, epoxy, etc., active bonding moieties, can be easily incorporated. This study renders the modified top side of CDs a promising support to develop new functionalities, preserve disc track trails, and make full advantage of CD reading technology.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

### Corresponding Author

*Phone: +34 963877342. Fax: +34 963879349. E-mail: amaquieira@qim.upv.es.

### Notes

The authors declare no competing financial interest.

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