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Development of Hapten-Linked Microimmunoassays on Polycarbonate Discs

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An amino-modified polycarbonate surface of compact discs is used to link haptens covalently and directly as an alternative to the classic protein−hapten conjugate adsorption coating strategy employed in immunoassays. The modified surface maintains its physical and optical properties, and a standard disk drive can then read the assay results. Advantages are evaluated, such as the use of a broader spectrum of coupling media including organic solvents that are inappropriate for proteins but necessary for some water-insoluble haptens and the bypassing of the synthesis and purification for protein conjugates. As proof of concept, competitive microimmunoassays were developed for chlorpyrifos, atrazine, and 2-(2,4,5-trichlorophenoxy)propionic acid (2,4,5-TP), in microarray format, obtaining detection limits of 37.2, 8.1, and 76 ng/L, respectively. The sensitivity was 1 order of magnitude better than that obtained for all the studied systems using hapten−protein conjugates adsorbed on polystyrene enzyme-linked immunosorbent assay (ELISA) plates and polycarbonate surfaces. Further, the influence of hapten structure and presentation on molecular recognition pattern is discussed. To our knowledge, this is the first time that microarray and compact disc technologies converge with this particular hapten immobilization mode. The great potential of the approach is demonstrated through the high-throughput capability of the disc in a range of analytical applications, as well as the inherent advantages of compact disc reading technology.

Immunoassays are valuable analytical methods with high sensitivity and selectivity. In the heterogeneous format, the surface of a polymer, normally polystyrene (PS), is used as the support on which probes are immobilized. The probe (hapten−protein conjugate for indirect format or an antibody for direct and sandwich formats) is usually immobilized on the surface by passive adsorption. However, covalent immobilization of probes is enhanced by background reduction, improved signal intensity associated with the target,[1] and a more stable probe layer.[2] In order to standardize and improve assay reproducibility, direct hapten linkage to the support is interesting since the hapten−protein conjugation procedure is quite variable.[3] This procedure allows for a broader range of coupling reactions including organic media that are inappropriate for proteins, thus eliminating methodological limitations related to the conjugation of extremely hydrophobic haptens.[4] Also, by directly binding the hapten to the support, it is not necessary to purify the conjugate, and a more direct and accessible hapten layer should be obtained since problems of protein folding are avoided, which would hinder further antibody recognition.

Carrier molecules different from proteins have been employed for hapten conjugation such as dextran, oligonucleotides, or biotin, but these do not eliminate all the drawbacks related to immunoassays based on protein conjugates particularly the need for synthesis and further purification steps.[3,5,6] Likewise, there are other approaches that employ haptens directly adsorbed to the assay surface, but they lack the advantages of covalent immobilization.[7,8]

Competitive immunoassays using direct and covalent hapten-functionalized supports have been developed mostly on two kinds of substrates: glass and plastic. Glass chips have been modified with different chemical groups to link a wide range of haptens for label-free detection. For example, Townsend et al. reported an immunoassay with a sensitivity of 2.4 μg/L for a morphine metabolite directly immobilized on a carboxylmethylated dextran derivatized surface plasmon resonance sensor chip;[9] Tschmelak et al. immobilized testosterone derivatives directly to a reflectometric interference spectroscopy transducer glass chip functionalized with an aminodextran layer to analyze testosterone in water samples with a limit of detection (LOD) of 0.2 ng/L.[10] However, these approaches are limited by the high prices of substrates and devices. More recently, the parallel analysis of 13 antibiotics in milk through a hapten microarray glass chip based on epoxy-activated poly(ethylene

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(1) Ivanova, E. P.; Wright, J. P.; Pham, D. K.; Brack, N.; Pigram, P.; Alekseeva, Y. V.; Demyashev, G. M.; Nicolau, D. V. *Biomed. Mater.* **2006**, *1*, 24–32.
(2) Ortega-Vinuesa, J. L.; Bastos-González, D.; Hidalgo-Alvárez, R. *J. Colloid Interface Sci.* **1995**, *176*, 240–247.
(3) Böcher, M.; Giersch, T.; Schmid, R. D. *J. Immunol. Methods* **1992**, *151*, 1–8.
(4) Estévez, M. C.; Kreuzer, M.; Sánchez-Baeza, F.; Marco, M. P. *Environ. Sci. Technol.* **2006**, *40*, 559–568.
(5) Tort, N.; Salvador, J.-P.; Eritja, R.; Poch, M.; Martínez, E.; Samitier, J.; Marco, M.-P. *Trends Anal. Chem.* **2009**, *28*, 718–728.
(6) Neokosmidi, A.; Ragoussis, V.; Zikos, C.; Paravatou-Petsotas, M.; Livaniou, E.; Ragoussis, N.; Evangelatos, G. *Talanta* **2008**, *74*, 539–546.
(7) Hofstetter, O.; Hofstetter, H.; Then, D.; Schurig, V.; Green, B. S. *J. Immunol. Methods* **1997**, *210*, 89–92.
(8) Fry, D. R.; Bobbitt, D. R. *Talanta* **2001**, *55*, 1195–1203.
(9) Townsend, S.; Finlay, W. J. J.; Hearty, S.; O'Kennedy, R. *Biosens. Bioelectron.* **2006**, *22*, 268–274.
(10) Tschmelak, J.; Kumpf, M.; Käppel, N.; Proll, G.; Gauglitz, G. *Talanta* **2006**, *69*, 343–350.

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glycol) surfaces and chemiluminescence detection has been reported by Kloth et al.[11]

As an alternative to glass, solid polymers offer attractive mechanical and chemical properties, presentations, low cost, fabrication facility and flexibility, and countless surface chemistry strategies to attach probes for immunoassaying. Reported in the literature is the covalent binding of haptens to PS microtiter wells bearing carbonyl and hydroxyl reactive groups by means of a disulfide spacer arm.[12] Commercially available maleic anhydride activated PS enzyme-linked immunosorbent assay (ELISA) plates have been used to immobilize an atrazine analogue, achieving effectiveness similar to *s*-triazine competitive immunoassays using protein conjugates passively adsorbed (IC_{50} values below 1 μg/L).[13] Further, an amino atrazine derivative has been covalently linked to a glutaraldehyde polymer network on the PS surface of microtiter plates, and high-sensitivity assays for terbutryn and atrazine have been developed (IC_{50} values of 0.13 and 0.26 μg/L, respectively).[14,15] Finally, in comparison to using protein conjugates, carboxylated haptens covalently linked to an aminoorganosilane attached to nitrated PS microtiter plates have proven useful to improve immunoassay sensitivity 10 times for 2,4-dichlorophenoxyacetic acid (2,4-D) (IC_{50} 7 μg/L instead of 70 μg/L) and 15 times for atrazine (IC_{50} 0.8 μg/L instead of 12 μg/L).[16]

All these approaches were developed on modified PS surfaces, resulting in highly reproducible hapten presentation, long-term storage ability of the coated surfaces, and improved analytical performances of the assays.

Chemical and physical processes have also been examined in the covalent attachment of proteins and nucleic acids to polycarbonate (PC) surfaces. Bora et al.[17] described the activation of PC plates with a photolinker to immobilize enzymes through their amino groups. Witek et al.[18] reported the photoactivation of PC microchips through UV radiation, generating carboxylate groups for DNA immobilization and purification. Noncontact printing of semicarbazide-functionalized silica nanoparticles on PC sheets for peptide micropatterning was developed by Carion et al.[19] Lastly, moldable PC granules tainted with dye molecules through a poly(ethylene glycol) linker demonstrated an effective binding of antidye monoclonal antibodies according to Najmabadi et al.[20]

The compact disc has been used as microarraying support, PC is the base substrate, given its mechanical and optical properties, the low cost of mass production, the possibility of surface functionalization, and the use of a standard drive as detector.[21,22] PC chemical modification to obtain an alkaline hydrolyzed surface to develop DNA microarrays on standard compact discs was patented by Remacle et al.,[23] while La Clair and Burkart reported on the phosphorylation of the PC surface to attach hydroxyl-ended ligands[24] as well as the spray-coating of a biotin label involving a poly(ethylene glycol)−polycarbonate tail on PC discs.[25] Most of these methods have limitations since they modify the optomechanical properties of the surface. Indeed, chemical reactions on discs must be carefully handled as PC can be damaged with organic solvents. Thus, Li and co-workers[26,27] reported a high surface density of carboxylic acid groups on PC discs using a UV/O_3 treatment that permitted the subsequent immobilization of biotin, oligonucletotides, and immunoglobulins by covalent linkage.

Our research team has approached disc surface modifications both by depositing a new material and by chemically functionalizing the bulk polymers. Regarding the first option, low-reflectivity discs (L-CDs) were successfully spin-coated with a hydroxy-modified poly(methyl methacrylate) (PMMA) polymer and then treated with an organosilane compound to produce an isocyanate-ended PMMA surface for oligonucleotide covalent anchoring.[28] Spin-coating PS on the gold surface of L-CDs has also been studied to develop microimmunoassays for chlorpyrifos, achieving better optical resolution and sensitivity than on untreated PC surfaces.[29]

Further developments on discs have involved PC nitration and reduction along with chloromethylation reactions to obtain amino- and thiol-functionalized surfaces, respectively. These modified surfaces retain the original mechanical and optical disk properties, and their advantages were highlighted in the SNP discrimination using covalently attached 21 base long oligomers.[30]

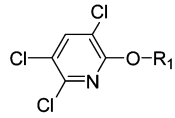
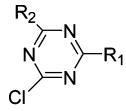
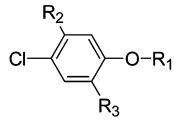
Considering the advantages of attaching haptens directly on polymers and the potential of disc technology for assaying, in this paper we describe a novel approach based on the direct covalent immobilization of haptens on transparent aminated PC surfaces compatible with the compact disc reading for immunochemical determination of agrochemical residues (chlorpyrifos, atrazine, and 2,4,5-TP) in water as proof of concept. Moreover, the influence of hapten structure and presentation on the molecular recognition pattern is examined to provide evidence supporting the improved performances obtained with our methodology.

MATERIALS AND METHODS

Chemicals. The immobilization−printing buffer (10 mM sodium phosphate buffer, 150 mM NaCl containing glycerol at 5% (v/v), pH 7.2), immunoassay buffer (PBS-T: 10 mM sodium phosphate buffer, 150 mM NaCl, 0.05% (v/v) Tween 20, pH 7.2),

(11) Kloth, K.; Rye-Johnsen, M.; Didier, A.; Dietrich, R.; Märtlbauer, E.; Niessner, R.; Seidel, M. *Analyst* **2009**, *134*, 1433–1439.
(12) Niveleau, A.; Sage, D.; Reynaud, C.; Bruno, C.; Legastelois, S.; Thomas, V.; Dante, R. *J. Immunol. Methods* **1993**, *159*, 177–187.
(13) Böcher, M.; Sorensen, K. *Food Agric. Immunol.* **1994**, *6*, 155–161.
(14) Holthues, H.; Pfeifer-Fukumura, U.; Hartman, I.; Baumann, W. *Fresenius' J. Anal. Chem.* **2001**, *371*, 897–902.
(15) Holthues, H.; Pfeifer-Fukumura, U.; Sound, I.; Baumann, W. *J. Immunol. Methods* **2005**, *304*, 68–77.
(16) Kaur, J.; Boro, R. C.; Wangoo, N.; Singh, K. R.; Suri, C. R. *Anal. Chim. Acta* **2008**, *607*, 92–99.
(17) Bora, U.; Sharma, P.; Kumar, S.; Kannan, K.; Nahar, P. *Talanta* **2006**, *70*, 624–629.
(18) Witek, M. A.; Llopis, S. D.; Wheatley, A.; McCarley, R. L.; Soper, S. A. *Nucleic Acids Res.* **2006**, *34*, e74/1–e74/9.
(19) Carion, O.; Souplet, V.; Olivier, C.; Maillet, C.; Medard, N.; El-Mahdi, O.; Durand, J.-O.; Melnyk, O. *ChemBioChem* **2007**, *8*, 315–322.
(20) Najmabadi, P.; Ko, K.-S.; La Clair, J. J.; Burkart, M. D. *JALA* **2008**, *13*, 284–288.
(21) Morais, S.; Carrascosa, J.; Mira, D.; Puchades, R.; Maquieira, A. *Anal. Chem.* **2007**, *79*, 7628–7635.
(22) Morais, S.; Tortajada-Genaro, L. A.; Arnandis-Chover, T.; Puchades, R.; Maquieira, A. *Anal. Chem.* **2009**, *81*, 5646–5654.
(23) Remacle, J.; Alexandre, I.; Houbion, Y. U.S. Patent 177144, 2002.
(24) La Clair, J. J.; Burkart, M. D. *Org. Biomol. Chem.* **2003**, *1*, 3244–3249.
(25) La Clair, J. J.; Burkart, M. D. *Org. Biomol. Chem.* **2006**, *4*, 3052–3055.
(26) Li, Y.; Wang, Z.; Ou, L. M. L.; Yu, H.-Y. *Anal. Chem.* **2007**, *79*, 426–433.
(27) Li, Y.; Ou, L. M. L.; Yu, H.-Y. *Anal. Chem.* **2008**, *80*, 8216–8223.
(28) Bañuls, M. J.; González-Pedro, V.; Puchades, R.; Maquieira, A. *Bioconjugate Chem.* **2007**, *18*, 1408–1414.
(29) Tamarit-López, J.; Morais, S.; Puchades, R.; Maquieira, A. *Anal. Chim. Acta* **2008**, *609*, 120–130.
(30) Bañuls, M. J.; García-Piñón, F.; Puchades, R.; Maquieira, A. *Bioconjugate Chem.* **2008**, *19*, 665–672.

Table 1. Chemical Structures of the Haptens and Analytes Studied

	Compound	R_1	R_2	R_3
	Triclopyr	CH_2COOH		
	Chlorpyrifos	$PS(OCH_2CH_3)_2$		
	Hapten 2d	$NH(CH_2)_5COOH$	$NHCH(CH_3)_2$	
	Atrazine	$NHCH_2CH_3$	$NHCH(CH_3)_2$	
	2,4,5-T	CH_2COOH	Cl	Cl
	2,4,5-TP	$CH(CH_3)COOH$	Cl	Cl
	2,4-D	CH_2COOH	H	Cl
	2,4-DP	$CH(CH_3)COOH$	H	Cl
	2,4-DB	$(CH_2)_3COOH$	H	Cl
	MCPA	CH_2COOH	H	CH_3
	MCPP	$CH(CH_3)COOH$	H	CH_3
	MCPB	$(CH_2)_3COOH$	H	CH_3

and washing solutions were filtered through a 0.22 μm pore size nitrocellulose membrane from Whatman GmbH (Dassel, Germany) before use.

The structures of haptens used in this experiment are depicted in Table 1. Hapten triclopyr (3,5,6-trichloro-2-pyridyloxyacetic acid) was from Dow AgroSciences LLG (Indianapolis, IN); hapten 2d (*N*-(4-chloro-6-isopropylamino-[1,3,5]triazin-2-yl)-6-aminohexanoic acid) was prepared as previously described.[31] 2-(2,4,5-Trichlorophenoxy)propionic (2,4,5-TP), 2,4,5-trichlorophenoxyacetic (2,4,5-T), 2,4-D, 4-(2,4-dichlorophenoxy)butyric (2,4-DB), 2-(2,4-dichlorophenoxy)propionic (2,4-DP), 4-chloro-2-methylphenoxyacetic (MCPA), 4-(4-chloro-*o*-tolyloxy)butyric (MCPB) as well as 2-(2-methyl-4-chlorophenoxy)propionic (MCPP) acids, dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), *N*,*N*-dimethylformamide (DMF), Tween 20, 5 nm colloidal gold-labeled goat antirabbit immunoglobulin (GAR−Au), silver enhancer solutions, ovalbumin (OVA), cold water fish skin gelatin (GEL), and bovine blood hemoglobin (Hb) were supplied by Sigma-Aldrich (Madrid, Spain). Chlorpyrifos and atrazine standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Antichlorpyrifos BSA−C2-II and anti-2,4,5-TP R58 polyclonal sera were previously obtained and characterized by ELISA.[32,33] Antiatrazine sera KLH-2d was obtained in our laboratory by rabbit immunization with KLH-2d antigen. Sodium borohydride was provided by Acros Organics (Geel, Belgium). Hammarsten's casein (CAS) was purchased from E. Merck (Darmstadt, Germany). Note: All the chemicals should be handled following the corresponding material safety data sheets.

(31) Gascón, J.; Oubiña, A.; Ballesteros, B.; Barceló, D.; Camps, F.; Marco, M. P.; González-Martínez, M. A.; Morais, S.; Puchades, R.; Maquiera, A. *Anal. Chim. Acta* **1997**, *347*, 149–162.

(32) Brun, E. M.; Garcés-García, M.; Puchades, R.; Maquieira, A. *J. Agric. Food Chem.* **2005**, *53*, 9352–9360.

(33) Morais, S.; Casino, P.; Marín, M. L.; Puchades, R.; Maquieira, A. *Anal. Bioanal. Chem.* **2002**, *374*, 262–268.

Analytical Disc Preparation. Transparent PC dummy plastic surfaces (0.6 mm thick and 12 cm diameter) were from Cóndor CD (Madrid, Spain). The surface amination is depicted in Figure 1. First, the discs were aminated with slight modifications as previously described.[30] Briefly, the PC was first immersed into an aqueous solution of nitric and sulfuric acid (20% and 5% v/v, respectively). After 10 min, the disc was washed with Milli-Q water, and the nitro groups were reduced with sodium borohydride for 6 h at room temperature. Then, the disc was washed with ethanol and rinsed with water. The treated surfaces were characterized physicochemically by determining water contact angles and by attenuated total reflectance Fourier transform infrared spectra, showing a peak at the 3200−3500 cm^{-1} region in accordance with the presence of amine groups. Finally, scanning electron microscopy (SEM) images demonstrated the homogeneity and lack of imperfections on the surface after the chemical treatment, maintaining the optical and mechanical properties.

Haptens were coupled through their carboxylic acid group to the amine-terminated PC surface by the DCC/NHS coupling chemistry. To this end, 50 μmol of hapten together with 50 μmol (5.8 mg) of NHS and 50 μmol (10.3 mg) of DCC were dissolved in 0.4 mL of anhydrous DMF. The reaction mixture was incubated for 2 h in the dark at room temperature (RT) while shaking gently. The mixture was then centrifuged at 8000 rpm for 15 min to remove acyl urea precipitate. Afterward, the activated hapten solutions were prepared in the immobilization buffer at serial dilutions. Solutions were immediately dispensed on a 384-well plate for microarraying on the amino−PC surface as 50 nL drops with a noncontact arrayer (AD1500, Biodot Inc., Irvine, CA) in a 90% humidity environment. The drop diameter was approximately 500 μm. The coupling reaction was carried out at room temperature for 16 h under controlled humidity conditions. Finally, the

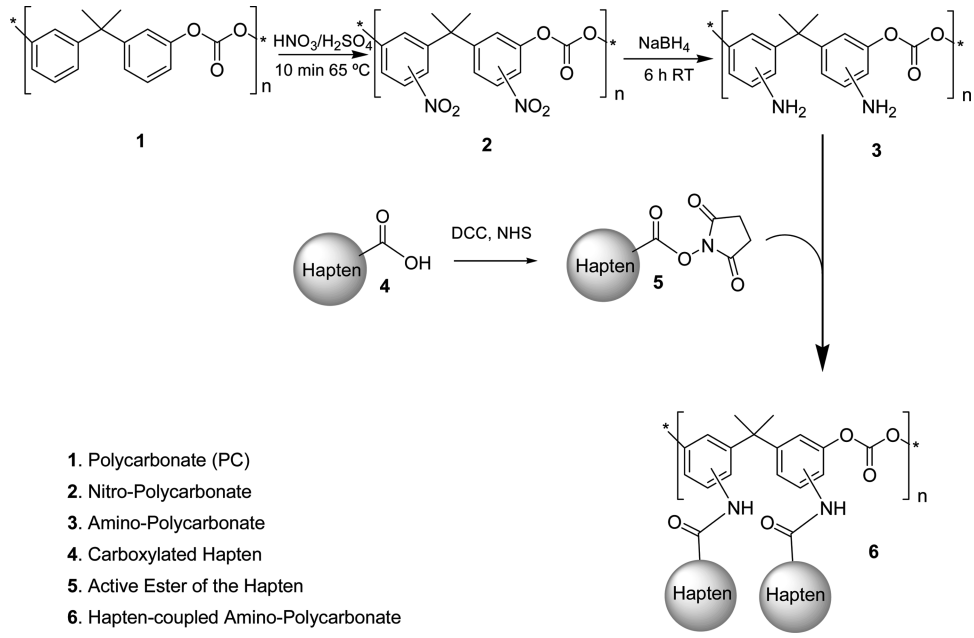


Figure 1. Scheme of direct chemical binding of carboxylated hapten molecules to the functionalized PC surface. Aqueous acid treatment of PC, followed by a hydride reduction, introduces amino groups on its surface. Activation of carboxylated haptens with DCC and NHS allows for their linkage to the amino−PC surface through an amide bond.

activated surface was washed with PBS for 1 min, rinsed with water, and dried by slight centrifugation.

Direct Hapten-Linked Microimmunoassay Protocol. The microimmunoassays on disc were based on an indirect competitive format. To this end, the arrays (8 × 8) printed on the hapten-linked disc (analytical disc) were coated with 50 μL of the specific antibody solution (C2-II diluted 1:800, KLH-2d diluted 1:2000 and R58 diluted 1:1000 in PBS-T for chlorpyrifos, atrazine, and chlorophenoxy acid assays, respectively) with or without the analyte and evenly distributed using 22 × 22 mm^2 glass coverslips (eight sample arrays per disc). After 10 min, the disc was washed with PBS-T, rinsed with deionized water, and dried by slight centrifugation. Next, 1 mL of GAR−Au solution (1:50 in PBS-T) was dispensed onto the disc and it was covered with a dummy surface. After 10 min at room temperature, the disc was washed, rinsed, and dried as before. To display the immunoreaction, the arrays were incubated with 800 μL of 1:1 (v/v) silver enhancer solution and the reaction was stopped by washing the disc with water after 8 min. The disc was dried as before, and later the results were read with a compact disc drive. The analytical disc was taped with two-faced adhesive onto a low-reflectivity disc (L-CD) (Figure 2). Low-reflectivity compact discs were purchased from Media Corp. (Tau-Yuan Shien, China). The reflectivity averaged 30.6% at 780 nm obtained by measuring the optical characteristics with a Dr. Schenk 146 scanner (Dr. Schenk GmbH, Planegg, Germany).

The resulting double-stacked disc was 1.8 mm wide, and the immunoreaction products formed on the analytical disc were in contact with the metallic side of an L-CD so as to achieve the best spatial and optical resolution. A conventional optical disk drive (Premium, Plextor America, Fremont, CA) was used as the detector; the management of the CD drive and data analysis is described elsewhere.[21] Inhibition curves were mathematically analyzed by fitting experimental results to a sigmoidal four-parameter logistic equation.

Figure 2. Hapten-linked microimmunoassays on compact discs. On the left, the analytical disc contains eight segmented microarrays (8 × 8). On the right, a semitransparent low-reflectivity compact disc (L-CD) overlaps part of the analytical disc.

Protein Conjugate Microimmunoassay Protocol. To develop microimmunoassays based on the indirect immobilization of haptens by means of carrier proteins, ovalbumin, casein, gelatin, and hemoglobin were conjugated to triclopyr by the active ester method. To this end, 100 μL of 250 mM activated triclopyr solution in DMF was slowly added to 15 mg of protein dissolved in 900 μL of 100 mM carbonate buffer (pH 9.6). The reaction mixtures were stirred for 2 h at RT, then centrifuged to remove any precipitate, and finally purified by gel exclusion chromatography on desalting columns (D-Salt, Pierce, Rockford, IL) using PBS (10 mM, pH 7.4) as the eluent. The hapten/protein molar ratios were 19:1, 37:1, 25:1, and 51:1 for OVA, CAS, GEL, and Hb conjugates, respectively, determined by ultraviolet spectroscopy, measuring the absorbance at 220 and 295 nm.

The conjugates were diluted in 100 mM carbonate buffer (pH 9.6) with 10% (v/v) glycerol. Solutions were microarrayed on the

L-CD PC surface with the noncontact arrayer as previously described. The discs were incubated at 4 °C for 16 h under humid conditions. Finally, the discs were washed with PBS for 1 min, rinsed with water, and dried as before. After that, the discs were ready to develop chlorpyrifos microimmunoassays as described above.

Dual-Polarization Interferometry Measurements. Dual-polarization interferometry (DPI) was used to characterize the layers of biomolecules immobilized on a sensor chip surface. The sensor chip is a dual-slab waveguide made of an upper sensing waveguide and a lower optical waveguide. The physical principle of this technique was described by Cross et al.[34] Briefly, laser light is switched between two polarizations: transverse magnetic (TM) and transverse electric (TE); the light travels along the sensor chip and, as it exits it, produces interference fringe patterns in the far field which are detected by a CCD camera.

DPI was used to obtain information about nonspecific adsorption of chlorpyrifos on a triclopyr layer immobilized on aminated silicon oxynitride chips (FB80 Amine, Farfield Sensors Ltd., Crewe, U.K.) using two procedures: direct covalent coupling and protein conjugate adsorption. The measurements were taken with the Analight Bio200 dual-polarization interferometer (Farfield Sensors Ltd., Crewe, U.K.). To this end, triclopyr and ovalbumin−triclopyr conjugate were independently loaded in separated channels of the sensor chip after calibration of the system with ethanol and water. Previously activated triclopyr with DCC and NHS was passed through channel 1 at 320 mg/L for 30 min in two sequential injections (20 and 10 min); OVA−triclopyr passed through channel 3 at 3.28 mg/L for 30 min (20 and 10 min). After rinsing with PBS, chlorpyrifos at 2 mg/L (maximum aqueous solubility) was simultaneously loaded into both channels for 20 min. The full experiment was set up at 24 °C and using PBS 1× as the loading and washing buffer.

The surface layer thickness and refractive index were determined from real-time measurements of optical phase changes in TM and TE. The immobilized mass, Γ (expressed in ng/mm^2), was later calculated from these data with the following equation:

$$\Gamma = d_f \frac{n_f - n_{buffer}}{dn/dc}$$

where d_f is the film thickness expressed in nm, n_f is the refractive index of the film, n_{buffer} is the refractive index of the buffer, and dn/dc is the specific refractive index increment. For the protein conjugate layer calculations, a dn/dc value of 0.18 mL/g was used since it is the value widely cited in the literature for proteins. For triclopyr and chlorpyrifos calculations, we chose a dn/dc value of 0.143 mL/g which corresponds to values obtained for small molecules such as urea.[35]

(34) Cross, G. H.; Reeves, A.; Brand, S.; Swann, M. J.; Peel, L. L.; Freeman, N. J.; Lu, J. R. *J. Phys. D: Appl. Phys.* **2004**, *37*, 74–80.
(35) Tumolo, T.; Angnes, L.; Baptista, M. S. *Anal. Biochem.* **2004**, *333*, 273–279.

RESULTS AND DISCUSSION

Hapten Linkage to Disc. The coupling of haptens to an amino−PC disc surface was considered for chlorpyrifos as a model system first by examining the conditions of activation and immobilization of the hapten triclopyr. The effect of reagent concentrations (DCC, NHS, and hapten) in the activation mixture was studied varying the initial level of all the compounds from 2.5 to 250 mM, maintaining the same molar ratio (1:1:1). After 2 h of activation, the supernatant was diluted to a final concentration of 1.25 mM and dispensed on the amino−PC surface. As seen in Figure 3A, the absolute signal increased sigmoidly with the activation reagent concentration up to 125 mM, probably because a higher hapten activated/nonactivated ratio is obtained at that point. Further, the effect of the molar ratio between NHS/DCC and triclopyr can be observed in Figure 3B; the maximum signal was reached at a molar ratio of 1.0. Under optimal conditions, the influence of pH on the immobilization−printing buffer was studied. To this end, several buffers with pH ranging from 4.0 to 8.0 were tested. The half-life of active NHS esters markedly decreases over pH 8.0,[36] and the pK_a of aniline conjugated acid is 4.6.[37] As shown in Figure 3C, the best results were obtained at neutral pH. This behavior might be attributed to an increment in the ratio of nonprotonated to protonated amino groups on the amino−PC as the pH increases, enhancing the nucleophility strength of the surface and, therefore, the hapten binding yield.

To verify the specificity and stability of the surface−hapten covalent bond, two negative control tests were performed. First, nonactivated triclopyr was incubated onto the amino−PC surface (negative control) and the resulting signal was below the detection limit (signal-to-noise ratio, S/N, <3). The response obtained incubating activated haptens on underivatized PC discs was also insignificant (S/N < 2). In addition, to confirm the covalent nature of the bond according to the amide linkage formed, the disc was washed with a high ionic strength solution (PBS 100 mM) to remove any hapten that might be physically or electrostatically adsorbed on the surface. The washing led to a slight decrease in the absolute signal. On average, 95% of the total signal was obtained, in comparison to that without washing.

On the other hand, the effectiveness of the washing step to remove adsorbed species was evaluated by treating discs in which coating conjugates were physically adsorbed. In this case, the washing caused a sharp decrease in the absolute signal, maintaining only 8% of the potential one, indicating that the high ionic strength solution successfully removed the adsorbed species from the disc, eliminating any nonspecific binding. From these results, we conclude that both disc functionalization and hapten activation are required to obtain an active and specific surface with covalently coupled haptens.

Competitive Immunoassays in Hapten-Linked Disc Format. *Chlorpyrifos Assay.* Competitive microimmunoassays for chlorpyrifos were performed by coupling triclopyr on discs as described in the Materials and Methods section. As the density of the immobilized hapten might affect the competition step, different dilutions of activated hapten, ranging from 25 to 1250

(36) Kim, H. S.; Kye, Y. S.; Hage, D. S. *J. Chromatogr., A* **2004**, *1049*, 51–61.
(37) Hall, N. F.; Sprinkle, M. R. *J. Am. Chem. Soc.* **1932**, *54*, 3469–3485.

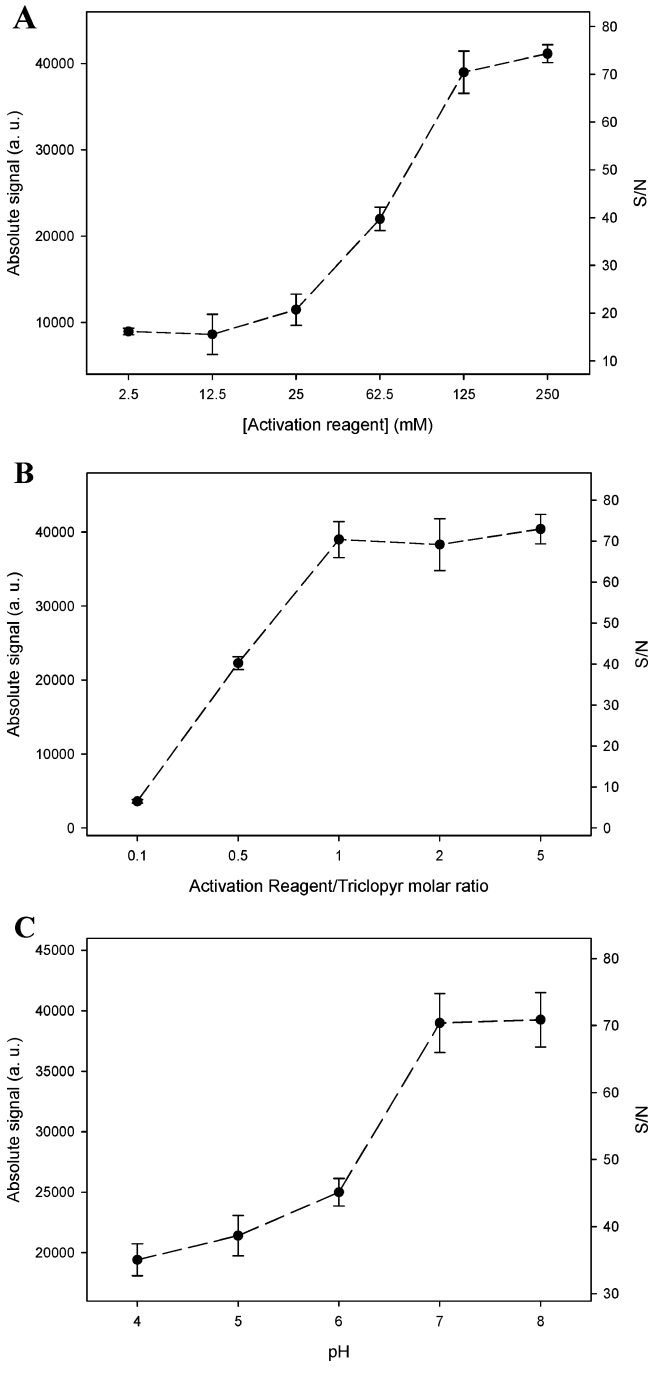


Figure 3. Absolute signal and S/N values (mean value ± standard deviation of 64 replicates) obtained from CD drive read-outs after a direct hapten-linked immunoassay in absence of analyte depending on the (A) activation reagent concentration (triclopyr, DCC, and NHS were equimolar at each concentration assayed), (B) molar ratio of DCC−NHS to triclopyr (125 μM) in the activation mixture, and (C) pH of immobilization solution (triclopyr at 125 μM).

μM, were dispensed onto amino−PC surfaces to assess the influence of this parameter on sensitivity. As illustrated in Figure 4A, the absolute signal for blank samples increases with the activated hapten concentration reaching a plateau at 125 μM. On the other hand, the sensitivity measured as IC_{50} varied from 450 to 774 ng/L for triclopyr concentrations between 125 and 1250 μM, respectively. It is worth mentioning that low hapten coating concentrations lead to more sensitive immunoassays (137 ng/L for 62.5 μM triclopyr). The

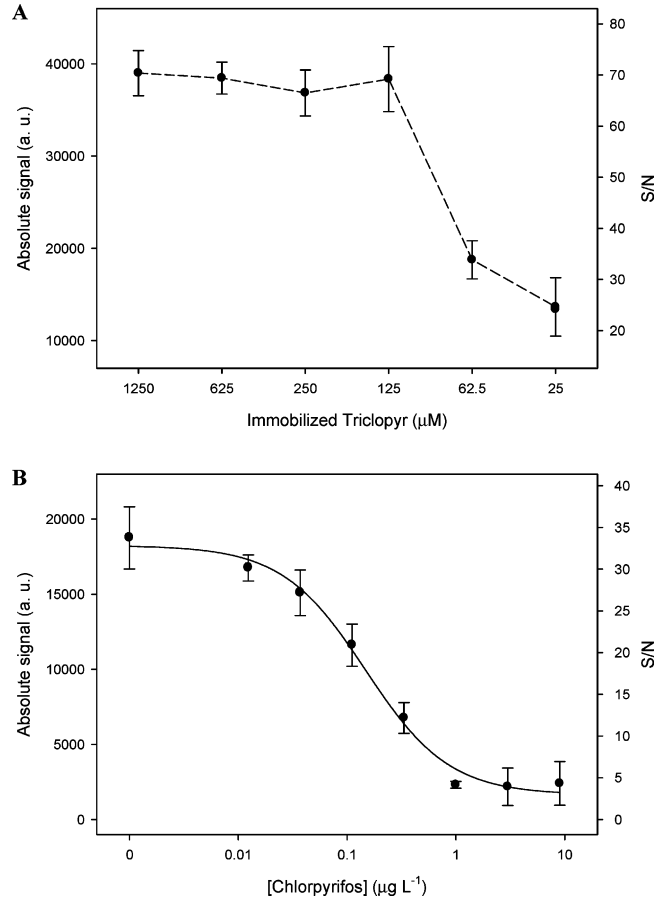


Figure 4. (A) Absolute signal and S/N values (mean value ± standard deviation of 64 replicates) obtained from CD drive read-outs after a direct hapten-linked microimmunoassay in absence of analyte for different concentrations of triclopyr coupled to the surface. (B) Calibration curve (mean value ± standard deviation of 512 replicates) for chlorpyrifos competitive immunoassay performed on disc surfaces with triclopyr linked at 62.5 μM.

criterion to select the optimal triclopyr concentration was based on reaching minimum IC_{50} (higher sensitivity) along with a suitable absolute signal. A S/N value statistically higher than 20 for blank samples (without analyte) was considered acceptable since it corresponds to a S/N of 10 at IC_{50}, according to the IUPAC definition for limit of quantification. Therefore, triclopyr at 62.5 μM was selected as the optimal hapten concentration to develop competitive immunoassays for chlorpyrifos. The standard calibration curve is shown in Figure 4B, representing the mean of eight curves performed on different days; in all, 512 spots per concentration of analyte were averaged. The intradisc relative standard deviation (RSD) for all the analyte concentrations ranged from 8.8% to 12.3%, whereas the mean interdisc RSD varied from 11.2% to 21.5%. As the analyte concentration increased, the signal-acquired intensity varied from the highest (absence of analyte) to background signal. The silver deposit resulting from the highest analyte concentration was minimal (S/N < 5), indicating a small degree of nonspecific response. The detection limit was 37.2 ng/L, and the dynamic range was between 47.0 and 588 ng/L, corresponding to 75% and 25% inhibition concentrations, respectively. The sensitivity (IC_{50}) was 137 ng/L, 1 order of magnitude in concentration better

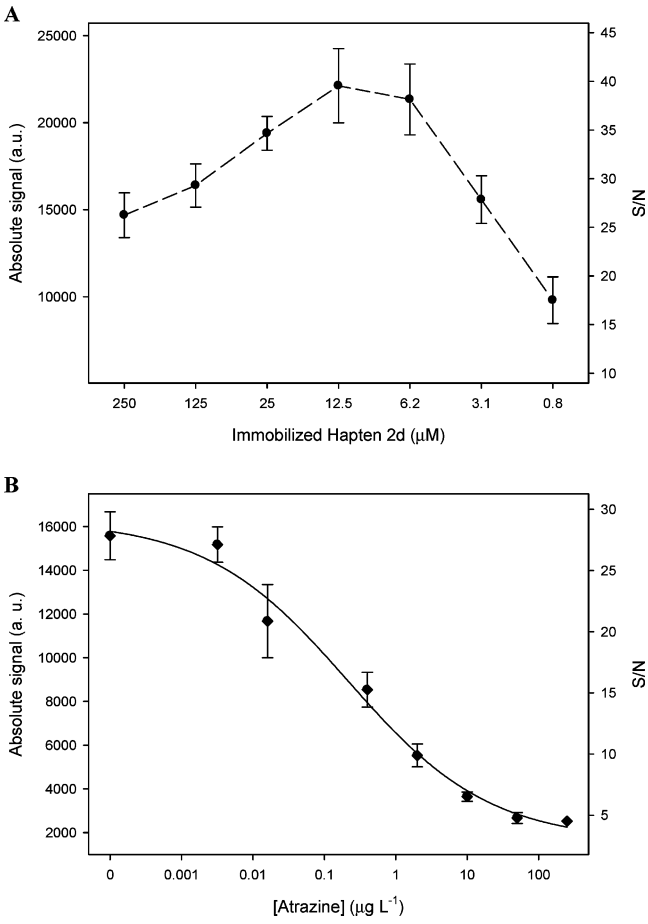


Figure 5. (A) Absolute signal and S/N values (mean value ± standard deviation of 64 replicates) obtained from the read-out of the compact disc drive after a direct hapten-linked microimmunoassay in absence of analyte for different concentrations of atrazine hapten 2d coupled to the surface. (B) Calibration curve (mean value ± standard deviation of 512 replicates) for atrazine competitive immunoassay performed on disc surfaces with hapten 2d coupled at 3.1 μM.

than that obtained with microimmunoassays using OVA–triclopyr conjugates immobilized by passive adsorption on underivatized PC surfaces.[21,29]

Atrazine Assay. A microimmunoassay for atrazine was developed in order to ascertain if these results were related exclusively with the triclopyr/chlorpyrifos system or if this is a general trend. To this end, hapten 2d (see Table 1) was covalently attached onto aminated dummy discs following the methodology described herein. As shown in Figure 5A, S/N values for blank samples increased as the 2d concentration decreased up to 12.5 μM. This might be associated with the poor solubility of the hapten in aqueous buffers.

In contrast to proteins, a range of available PC-compatible solvents can be used to dissolve haptens for conjugation. Taking advantage of this, activated hapten 2d (125 mM) was dissolved in an immobilization buffer containing DMF and dispensed onto the aminated surface as described before. As a trade-off between maximum hapten solubility and maintaining optical disk properties, an immobilization buffer containing 67% DMF (v/v) was used for hapten coupling. In terms of absolute signal and sensitivity, the results were similar to those obtained with the aqueous immobilization buffer. This behavior may be attributed to the formation of NH hydrogen bonds between two 2d molecules when closely packed. This interaction would hinder the subsequent antibody recognition, given the high dependence of specific hapten–antibody interaction on hydrogen-bond formation. These observations are in good agreement with those reported by Pozharski et al.[38]

In terms of sensitivity, the best results were obtained using hapten 2d at 3.1 μM, reaching an acceptable absolute signal and S/N (S/N statistically higher than 20), with a detection limit of 8.1 ng/L, an IC_{50} of 213 ng/L, and a dynamic range between 35 and 10 300 ng/L. The calibration curve is depicted in Figure 5B, as the mean of eight curves performed on different days. The intradisc RSD for all the analyte concentrations ranged from 9.5% to 14.1%, while the mean interdisc RSD varied from 13.1% to 20.5%.

The sensitivity of this methodology was quite high and similar to reported microimmunoassays using atrazine 2d–protein conjugates immobilized by adsorption on PC discs[39] and PS ELISA plates.[31]

Effect of Hapten Structure on Sensitivity. The immunoassay sensitivity is dependent on the affinity of the antibody for the analyte compared to that present for the hapten linked to the surface. In accordance with the heterology principle, antibody affinity to conjugate hapten should be slightly lower than that for the analyte to obtain optimum competition conditions.[40] The affinity balance can be modulated by changing either the chemical structure of the hapten or the spacer arm length. To conduct the experiment on the direct hapten-coupled surfaces, a set of eight chlorophenoxy acid compounds (2,4-D, 2,4-DB, 2,4-DP, 2,4,5-T, 2,4,5-TP, MCPA, MCPP, and MCPB; structure in Table 1) were assayed at different immobilization densities. All these compounds present a carboxylate moiety to couple to the aminated surface. The microimmunoassays for four of the eight analytes (2,4-D, 2,4-DB, MCPA, and MCPB) did not reach an appropriate S/N (S/N < 20). The remaining haptens (2,4-DP, 2,4,5-T, 2,4,5-TP, and MCPP) gave results with acceptable S/N values. The trend of the absolute signal was similar to that obtained for chlorpyrifos; the signal decreases as the coating concentration increases, indicating that hydrogen bonds were not formed as was the case of atrazine. Also, the microimmunoassay response depends on the chemical structure of the haptens, in particular the R_1 substituent. Thus, only haptens with an isopropionic chain (2,4-DP, 2,4,5-TP, and MCPP) or with three chlorine atoms on the aromatic ring (2,4,5-TP and 2,4,5-T) generated an appropriate response.

The relationships between the antibody affinity and the hapten structure might explain the above observations. Indeed, as the R-58 serum was raised from a rabbit immunized with 2,4,5-TP–KLH conjugate,[33] maximum immunological recognition might be expected to occur against 2,4,5-TP or an antigen with a similar chemical structure.[41] In relation to immobilized hapten orientation, the linking bridge is not so exposed to antibody binding, so the epitope recognized by the antibody molecule should be located

(38) Pozharski, E.; Moulin, A.; Hewagama, A.; Shanafelt, A. B.; Petsko, G. A.; Ringe, D. *J. Mol. Biol.* **2005**, *349*, 570–582.
(39) Morais, S.; Tamarit-López, J.; Carrascosa, J.; Puchades, R.; Maquieira, A. *Anal. Bioanal. Chem.* **2008**, *391*, 2837–2844.
(40) Schneider, P.; Hammock, B. D. *J. Agric. Food Chem.* **1992**, *40*, 525–530.
(41) Zhang, Q.; Wang, L.; Ahn, K. C.; Sun, Q.; Hu, B.; Wang, J.; Liu, F. *Anal. Chim. Acta* **2007**, *596*, 303–311.

in the aromatic part of the herbicide.[42] However, in our case, both parts of the hapten structure, the aromatic ring and the linking bridge, are crucial in the recognition step.

The hapten used to develop competitive immunoassays for 2,4,5-TP on discs was selected based on its analytical response and sensitivity. In accordance with the heterology principle, coating haptens with lower affinity for the antibody should enhance sensitivity. Also, an acceptable response as defined above (S/N > 20) is needed for proper quantification. Immobilization of 2,4-DP at 125 μM gave microimmunoassays with a detection limit of 76 ng/L, an IC_{50} of 163 ng/L, and a dynamic range between 116 and 336 ng/L for 2,4,5-TP. As before, the sensitivity of the microimmunoassay for 2,4,5-TP was 5 times higher (lower IC_{50}) than the sensitivity values obtained with microimmunoassays on PC discs and ELISA plates, using adsorbed protein conjugates.[21,33]

To evaluate the effect of heterology on assay sensitivity, a competitive assay for 2,4,5-TP was developed immobilizing 2,4,5-TP at 125 μM, reaching an IC_{50} of 6.20 μg/L. This value was higher than that obtained with hapten 2,4-DP (0.16 μg/L), being in good agreement with the heterology principle.

Regarding the selectivity of the assay, MCPB was the main interfering compound (13.5%; IC_{50} 1200 ng/L), and weak cross-reactivity (<2%) was observed for the set of chlorophenoxy acid herbicides tested. Under the optimized conditions, therefore, the assay for 2,4,5-TP was as specific as the ELISA plate format.[33]

Covalent Linkage versus Passive Adsorption. The results obtained clearly demonstrate an improvement in assay sensitivity when the hapten is immobilized directly to the assay support compared to its immobilization through ovalbumin conjugates. It is known that different carrier proteins can lead to different analytical performances of the immunoassays,[43] so it is necessary to check that the improvement in sensitivity achieved with the direct coupling of the hapten is maintained in comparison with any conjugate regardless of the protein employed. For this, a set of hapten−protein conjugates were prepared using triclopyr as the model hapten. OVA, CAS, GEL, and Hb were chosen as the carrier proteins given their commercial availability, high purity, and their suitability to conjugate haptens by the active ester procedure; they are also well-defined in physicochemical terms and widely used in ELISA. Moreover, these proteins represent a broad range of physical and chemical protein structures.

Competitive microimmunoassays for chlorpyrifos were performed with protein conjugate discs coated with triclopyr equivalent concentrations ranging from 0.30 to 75.0 μM. The best results in terms of absolute signal and sensitivity were obtained using a 1.25 μM equivalent triclopyr concentration which resulted in IC_{50} values of 4.2, 13.0, 21.8, and 29.0 μg/L for OVA, CAS, GEL, and Hb conjugates, respectively. The slight differences in assay sensitivity among the tested conjugates reflected changes in the antibody−hapten affinity, the assay being more sensitive as the affinity decreased. In any case, the sensitivity obtained with direct hapten coupling was at least 30.6 times better than that using protein conjugates.

This might reflect a higher antibody affinity for the conjugates than for triclopyr directly linked to amino−PC discs given that, with protein conjugates, the amount of hapten needed to obtain an appropriate absolute signal is less than that of triclopyr to develop hapten-coupled surface assays. However, this trend is most likely related to differences in the epitope concentration present on the surface which, in turn, will determine the amount of antibody that is finally immobilized. In fact, the two procedures of hapten immobilization could lead to very different reaction yields. In the case of direct hapten coupling, the yield of the activation step does not have to be 100%, and even if this occurred, hapten deactivation during the immobilization step is possible since an aqueous buffer is used in this case. Therefore, the printing of higher hapten concentrations on amino−PC surfaces will be required to reach the same epitope surface density as that of protein conjugates adsorbed on PC.

Dual-Polarization Interferometry Experiments. The improved sensitivity achieved with hapten covalently linked assays, as compared to protein conjugate based microimmunoassays, may be attributable to the chemical structures of the analytes and haptens tested. Thus, the hydrophobic nature of chlorpyrifos, atrazine, and 2,4,5-TP, together with the hydrophobic character of the covalently hapten-coupled surfaces, may cause analyte enrichment on the aminated surface. This effect would be greater than on surfaces loaded with protein conjugates where an analyte−hapten interaction would be hindered by the hydrophilic domains of proteins. To elucidate the aforementioned assumption, DPI measurements were performed on amine-modified chips to analyze the nonspecific adsorption of chlorpyrifos. Thus, chlorpyrifos at 2 mg/L (maximum solubility in water) was passed through two channels of the chip, one with triclopyr covalently immobilized and the other channel with OVA−triclopyr conjugate passively adsorbed in amounts similar to those employed in disc assays. DPI data highlighted an analyte enrichment phenomenon on both hapten surfaces, but the width of the chlorpyrifos layer was significantly greater in the channel in which OVA−triclopyr was adsorbed (Figure 6), which meant that the improvement in assay sensitivity was not due to this phenomenon. The difference in the nonspecific adsorption of chlorpyrifos in each case could be related to changes in surface roughness. Indeed, a surface with large immobilized molecules, such as proteins, will be a more effective surface for adsorbing small molecules like chlorpyrifos.

Other effects, such as the valency of antibody−ligand interaction, might explain the differences in sensitivity between assays in which the hapten was directly (covalent) or indirectly (passive) linked to the surface. Indeed, it is well-known that assay sensitivity increases as long as a monovalent, not bivalent, antibody−antigen interaction predominates. Monovalent fragments from whole antibodies or decreased hapten−protein ratios of immobilized conjugates were found to increase sensitivity several orders of magnitude due to the predominant monovalent relationship between antibody and antigen.[44]

(42) Trau, D.; Theuerl, T.; Wilmer, M.; Meusel, M.; Spener, F. *Biosens. Bioelectron.* **1997**, *12*, 499–510.
(43) Brun, E. M.; Garcés-García, M.; Bañuls, M. J.; Gabaldón, J. A.; Puchades, R.; Maquieira, A. *Environ. Sci. Technol.* **2005**, *39*, 2786–2794.

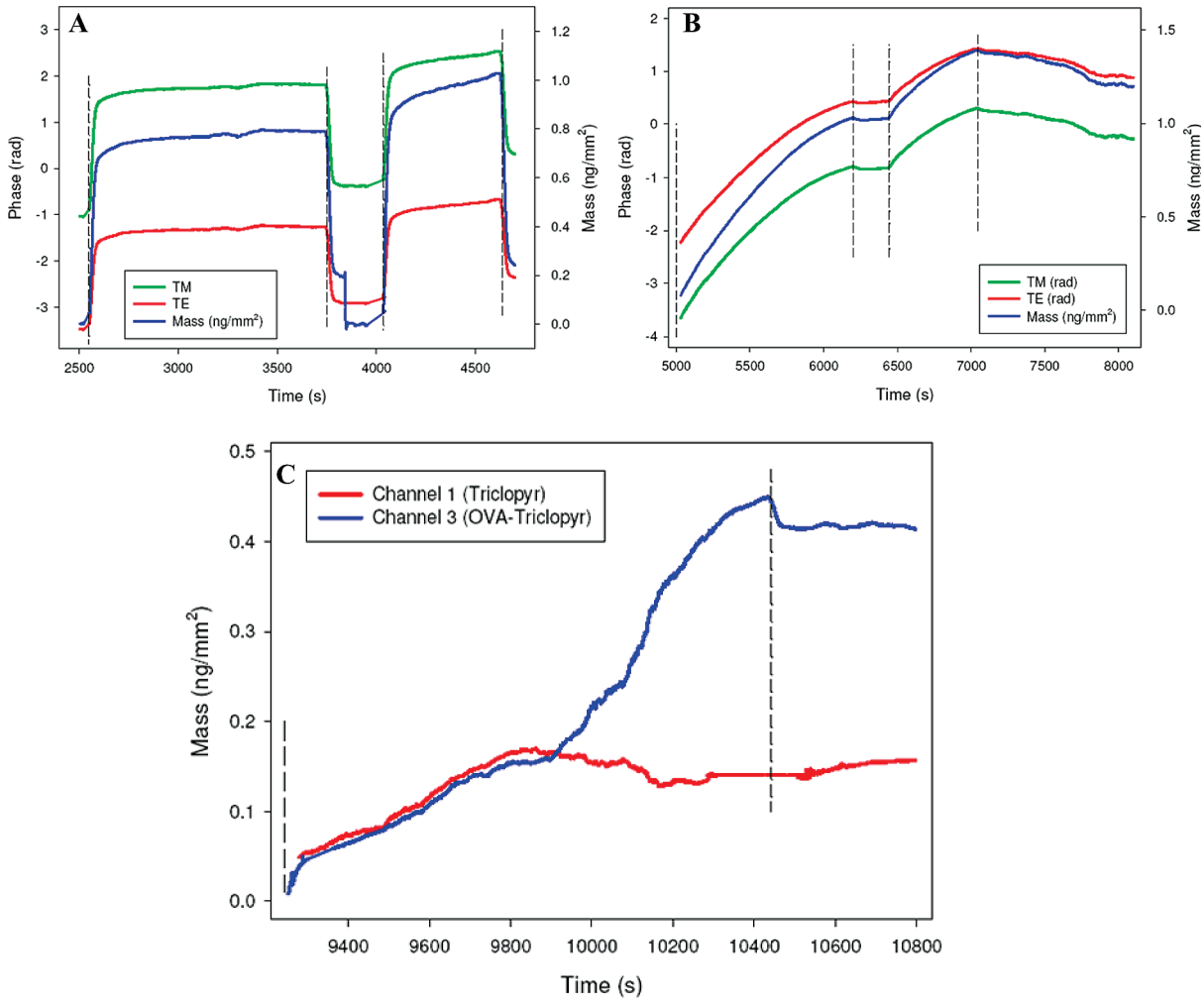


Figure 6. DPI read-outs and the calculated immobilized mass obtained for (A) triclopyr, (B) OVA−triclopyr, and (C) chlorpyrifos loads. Vertical dash lines indicate the start and end time of each load. Mass layers of 0.345 and 1.082 ng/mm^2 were obtained through triclopyr and OVA−triclopyr injections, respectively. After the chlorpyrifos load, a mass layer increment of 0.158 and 0.401 ng/mm^2 was observed in the covalently coupled triclopyr and adsorbed protein conjugate channels, respectively.

In this sense, the different methods of hapten immobilization lead to differences in hapten presentation, affecting antibody-binding profiles and hence assay sensitivity.[45] Since each protein molecule possesses multiple hapten moieties, a bivalent interaction between antibody and antigen is favored, leading to high-avidity bivalent surface attachment of whole antibodies. This bivalent interaction may be less pronounced when the hapten is directly linked and more sensitive immunoassays are achieved.[9]

CONCLUSIONS

This is the first study in which microimmunoassays are performed linking haptens covalently to aminated surfaces so as to detect low-abundant compounds using compact disc technology. The findings reported herein add to the literature on direct hapten-coupled systems and their specific achievements. The physical and optical properties of the functionalized support were maintained as was hapten bioavailability. This approach will also be useful for applications on other microarraying substrates, specifically those made of polymeric materials. Since no cross-linkers are needed to bind haptens to the disc surface, the hapten is directly linked to the support. This provides a clean and reproducible hapten-coupled surface that may elucidate immunochemical theoretical knowledge in terms of heterology studies and approaches concerning the influence of hapten structure on antibody affinity, as those carried out with chlorophenoxy acid compounds.

Direct hapten binding on a polymeric surface such as PC should also facilitate the use of many reagents and reactions. While easily correcting drawbacks related to the attachment of certain haptens, this approach may be used with other immobilization protocols and assaying methodologies that cannot be applied to proteins. Likewise, this approach eliminates the need for synthesis and further purification of the hapten−protein coating conjugates.

One of the major conclusions of this study is related to assay sensitivity for all haptens tested. The sensitivity obtained was at least 1 order of magnitude in concentration better than that for the assays based on traditional hapten−protein conjugate adsorption formats developed on PS ELISA plate or on PC disc surfaces. The improvement in assay sensitivity is due to differences in the

(44) Gerdes, M.; Meusel, M.; Spener, F. *J. Immunol. Methods* **1999**, *223*, 217–226.
(45) Jung, H.; Yang, T.; Lasagna, M. D.; Shi, J.; Reinhart, G. D.; Cremer, P. S. *Biophys. J.* **2008**, *94*, 3094–3103.

spatial distribution of the epitopes on the surface which, in turn, changes the valency of antibody−hapten interaction.

It is worth mentioning that disc microarraying permits the simultaneous binding of different probes to an inexpensive and readily available support to further develop multiplexed immunoassays in a single sample, for example, for simultaneous detection of drugs, hormones, or cancer markers. In addition, the absence of carrier proteins for hapten immobilization should allow for the extension of multianalysis ability of a single disc, since there are no interference effects between a particular antibody and conjugation proteins of different immunochemical systems.

The high-throughput capability of the disc, as well as the inherent advantages of compact disc reading technology, such as ubiquity and low cost, and the outstanding characteristics described herein, contribute to making this methodology of great potential in a wide range of analytical applications.

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SUPPORTING INFORMATION AVAILABLE

Immunoassay scheme and data analysis, and analysis of real samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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