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High-Throughput Multiplexed Competitive Immunoassay for Pollutants Sensing in Water

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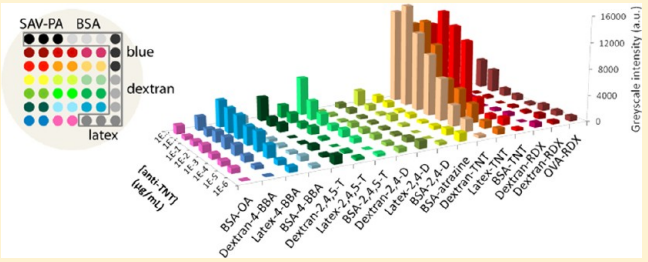
Supporting Information

ABSTRACT: The present study described the development and evaluation of a new fully automated multiplex competitive immunoassay enabling the simultaneous detection of five water pollutants (okadaic acid (OA), 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine (atrazine), 2.4-dichlorophenoxy-acetic acid (2,4-D), 2,4,6-trinitrotoluene (TNT), and 1,3,5-trinitroperhydro-1,3,5-triazine (RDX)). The technology is taking advantage of an optical-clear pressure-sensitive adhesive on which biomolecules can be immobilized and that can be integrated within a classical 96-well format. The optimization of the microarray composition and cross-reaction was performed using an original approach where probe molecules (haptens) were conjugated to different carriers such as protein (bovine serum albumin or ovalbumin), amino-functionalized latex beads, or dextran polymer and arrayed at the surface of the adhesive. A total of 17 different probes were then arrayed together with controls on the adhesive surface and screened toward their specific reactivity and cross-reactivity. Once optimized, the complete setup was used for the detection of the five target molecules (less than 3 h for 96 samples). Limits of detection of 0.02, 0.01, 0.01, 100, and 0.02 μg L^{-1} were found for OA, atrazine, 2,4-D, TNT, and RDX, respectively. The proof of concept of the multiplex competitive detection (semiquantitative or qualitative) of the five pollutants was also demonstrated on 16 spiked samples.



The presence of toxic contamination traces in water having adverse effect on human health and wildlife is an ongoing concern. These pollutants can be pesticides/herbicides coming from intensive agricultural activities, industrial side products, pharmaceutical chemistry inherited compounds (drugs, antibiotic), explosives molecules from terrorists and military activities, or toxins produced by marine animals or phytoplankton blooms. Okadaic acid (OA), for example, is a diarrheic toxin produced by toxicogenic dinoflagellates. This phycotoxin accumulates in the digestive glands of shellfish without a toxic effect to its host. Nevertheless, as shellfish is a part of human alimentation chain, this toxin accumulation can be harmful, causing diarrheic shellfish poisoning (DSP), which is characterized by gastrointestinal disorders.[1]

Explosive molecules, such as 2,4,6-trinitrotoluene (TNT) or 1,3,5-trinitroperhydro-1,3,5-triazine (RDX), are also considered as toxic compounds since they may cause severe health problems in both animals and humans populations.[2,3] These troubles include anemia, abnormal liver function, cataract development, and skin irritation. Besides, the Environmental Protection Agency (EPA) has determined that TNT and RDX are possible human carcinogens. TNT and RDX pollute the environment through the contamination of waste waters and solid wastes coming from either the explosive industry and the related bombing activity or the bomb recycling. Thus, different military and terrorist activities worldwide have resulted in extensive contamination of soil and groundwater by TNT or RDX.[4]

Atrazine and 2,4-dichlorophenoxyacetic acid (2,4-D) are organochlorine herbicides used in global intensive agriculture. Because of their widespread use and persistence, their accumulation in soil and groundwater became a real public health issue. In the European Union, concentration limits for individual pesticides and the total pesticide content in drinking water are 0.1 and 0.5 μg L^{-1}, respectively.[5] In the U.S., the EPA sets the maximum allowed concentrations for common pesticides such as atrazine to 3 μg L^{-1}.[6]

The detection of these different types of molecules is an important environmental, security, and health concern for the global community. The simultaneous presence of more than one of these pollutants in water is now often performed, and multiparametric analytical tools are required. Currently, detection of herbicides is mostly performed by chromatography (gas chromatography (GC), high-pressure liquid chromatography (HPLC)), mass spectrometry (mass spectrometry-time-of-flight (MS-TOF), mass spectrometry-ion trap (MS-IT), mass spectrometry-quadrupole (MS-Q)), and capillary electropho-

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resis (CE) methods. For instance, the EPA recommends GC/MS and GC/electron capture detection (ECD) for analysis of atrazine and 2,4-D in water samples.[7−9] Nevertheless, these methods are still time-consuming and rely on sophisticated equipments and laborious sample preparation. In order to cope with these issues, there is a growing demand from the environmental monitoring community for inexpensive and sensitive analytical devices that are reliable, rapid, and capable of high-throughput testing. Among the different types of sensors recently developed for this purpose (electrochemical sensors, mass sensors, and optical sensors[10−13]), immunoassay like systems appear to be the most popular thanks to their high selectivity and to the standardization and automation possibilities of their protocols. Particularly, monoparametric immunosensors have been proven to be valuable tools to detect trace amounts (ppm to ppb) of specific contaminants such as pesticides, toxins, or explosives in environmental samples.[14,15] However, most of the previous systems dedicated to the detection of environmental pollutants were focused on few toxicants only and rarely integrating different pollution sources (i.e., intensive agricultural activities, industrial side products, pharmaceutical chemistry, military activities, and phytoplanktons).[16−19] For example, Morais et al. recently described an on-disk multiplexed immunoassay for the detection of pesticides and antibiotics.[20] In a similar way Weller et al. described a "parallel affinity sensor array" dedicated to the direct and indirect immunodetection of contaminants in water thanks to a glass slide biochip.[21]

In the present communication, we report a new multiplex immunoassay for the simultaneous detection of environmental pollutants coming from intensive agricultural activities, military activities and phytoplankton blooms (atrazine, 2,4-D, RDX, TNT, and okadaic acid (OA)). For the development of this multiplex immunoassay, we have been screening 17 carrier-hapten conjugates toward their reactivity and cross-reactivity with specific antibodies. Using microarrays in a 96-well plate format enabled us to generate large amount of experimental data in highly controlled conditions, facilitating the optimization of the first five-plex competitive immunoassay. The operational multiparameter tool was demonstrated to be able to simultaneously detect atrazine, 2,4-D, RDX, TNT, and OA with limits of detection relevant to the EU and U.S. regulations.

EXPERIMENTAL SECTION

Pollutants, Antibodies, and Reagents. Polybead Amino Microspheres (1.00 μm) were purchased from Polyscience, U.S. Dextran Amino (500 000 Da) was supplied by Life Technologies, United Kingdom. BCIP/NBT, 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine (atrazine), 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 4-benzoylbenzoic acid (4-BBA), okadaic acid from *Prorocentrum concavum*, picrylsulfonic acid (TNBS) solution 1 M in H_2O, bovine serum albumin (BSA), and alkaline phosphatase labeled antimouse IgG (Fc specific) antibodies developed in goat were obtained from Sigma-Aldrich, France. 2,4,6-Trinitrotoluene (TNT) and RDX were purchased from LGC Standards, France. Atrazine-BSA and antiatrazine 6F36 monoclonal antibodies developed in mouse were supplied by Euromedex, France. Anti-RDX antibodies and anti-TNT A1 monoclonal antibodies developed in mouse were supplied by Strategic Diagnostics, U.S. Antiokadaic acid 7E1 monoclonal antibodies developed in mouse were purchased from Santa Cruz Biotechnology, U.S. Alkaline phosphatase-labeled antimouse IgG antibodies (H+L) developed in goat were supplied by Jackson Immuno-Research, U.S. LowCross-Buffer was purchased from Candor Bioscience, Germany. Anti-2,4-D E2G2 monoclonal antibodies were kindly provided by Dr. Milan Franek. RDX-ovalbumin (OVA-RDX) and dextran-RDX were kindly provided by Prof. Stephano Girotti.

Hapten-Carrier Conjugates Synthesis. In order to integrate in a microarray the small molecule hapten probes (about 300 g mol^{-1} for explosives and pesticides and 800 g mol^{-1} for toxins), conjugates of higher molecular weight were synthesized. Seven different haptens (three pesticides, atrazine, 2,4-D, and 2,4,5-T; two explosives, TNT and RDX; one toxin, okadaic acid; one control, 4-BBA) were conjugated to three different carriers (latex-amino, dextran-amino, and BSA). For the okadaic acid conjugate, BSA only was used on the basis of the good results obtained previously with this probe.[22]

For all haptens, the conjugation was performed by coupling the primary amine function of the carriers with the carboxylic acid function of the hapten after their carbodiimide activation. Briefly, the probes were activated via their carboxylic acid function by a pretreatment in 1,4-dioxane at the concentration of 1.36 g L^{-1} in the presence of 3.9 g L^{-1} *N*-hydroxysuccinimide and 14.8 g L^{-1} *N*,*N*′-dicyclohexylcarbodiimide. After an incubation time of 30 min, the dicyclourea precipitate was eliminated by centrifugation, and 20 μL of the supernatant were added to either (i) 500 μL of a 10 g L^{-1} BSA solution, (ii) 250 μL of a latex-amino beads solution, or (iii) 250 μL of a dextran-amino solution (2 μM). All carrier solutions were prepared in 0.1 M carbonate buffer, pH 11. The obtained solutions were then incubated under stirring for 2 h at room temperature for the coupling process to be completed. The formed BSA-conjugates were then separated from the nonreacted species on a desalting chromatography column (Sephadex G-25 M). The formed latex- and dextran-conjugates were separated from the nonreacted species by successive centrifugation/rinsing cycles using microcon 3000 columns. The conjugates were stored in 0.1 M acetate buffer, KCl 0.1 M, pH 5.5 at 4 °C.

These three carriers were used because of their difference in terms of available amino groups and to evaluate the possible cross-reactivity toward carrier. For BSA, there are 59 lysine residues and 30−35 are accessible for the coupling reaction.[23] The polybead amino microspheres contain an unknown number of NH_2, but they are believed to exhibit a high density of amino groups. The dextran amino contains 146 mol of NH_2/mol.

In order to characterize the conjugation, the protein conjugates were studied using UV or mass spectrometry, and the determined molar ratio for each pollutant is shown in Supporting Information 1.

Microarray Preparation. The obtained conjugate solutions were spotted with the addition of 0.5 g L^{-1} bromophenol blue as the spotting control. A volume of 0.8 nL of each solution were arrayed with a 450 μm spot to spot pitch in a 49-spots matrix format (7 × 7 array) on an optical clear pressure sensitive adhesive (TKL, AXO Science, France) using a piezoelectric spotter (sciFLEXARRAYER S3, SCIENION, Germany).

For the first part of the study (matrix I) concerning the screening of the conjugates and the selectivity tests, all the conjugates were deposited in duplicates, with triplicates of positive control (alkaline phosphatase labeled streptavidin), and triplicates of seven nonspecific interaction controls (BSA-4-BBA, latex-4-BBA, dextran-4-BBA, BSA, latex, dextran, and bromophenol blue).

For the second part of the study (matrix II) concerning the calibration of the test and the pollutants multiplex detection, the

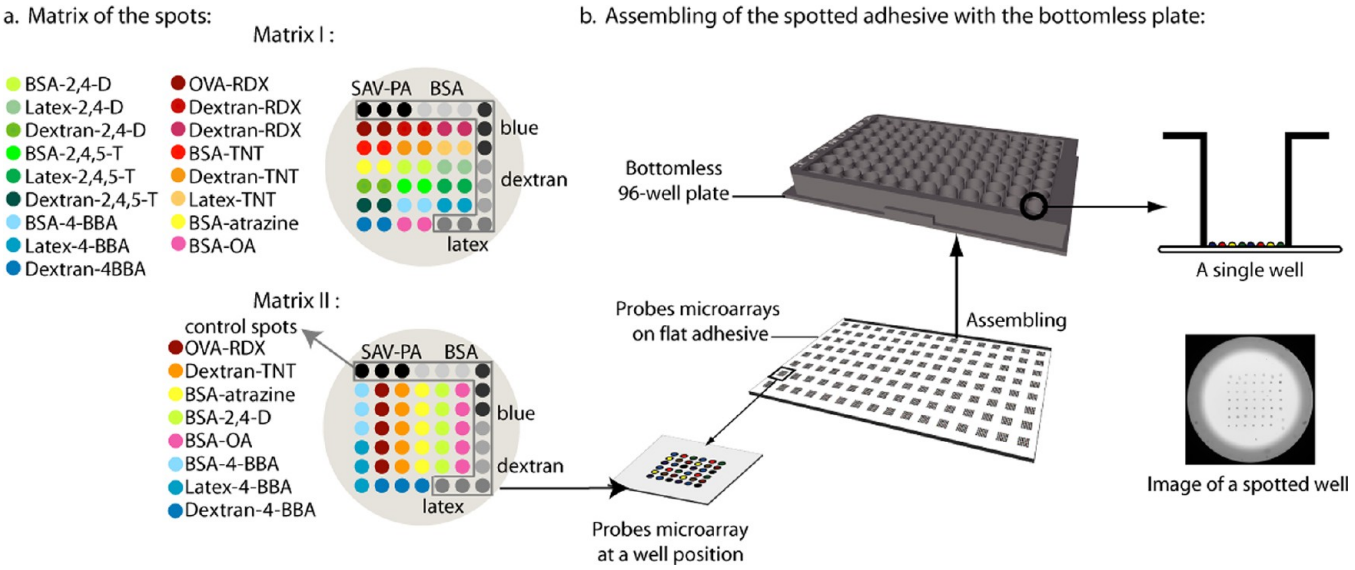

Figure 1. Competitive multiplex immunoassay setup: (a) composition of the different matrixes used and (b) principle of the adhesive microarray assembly with the bottomless plate.

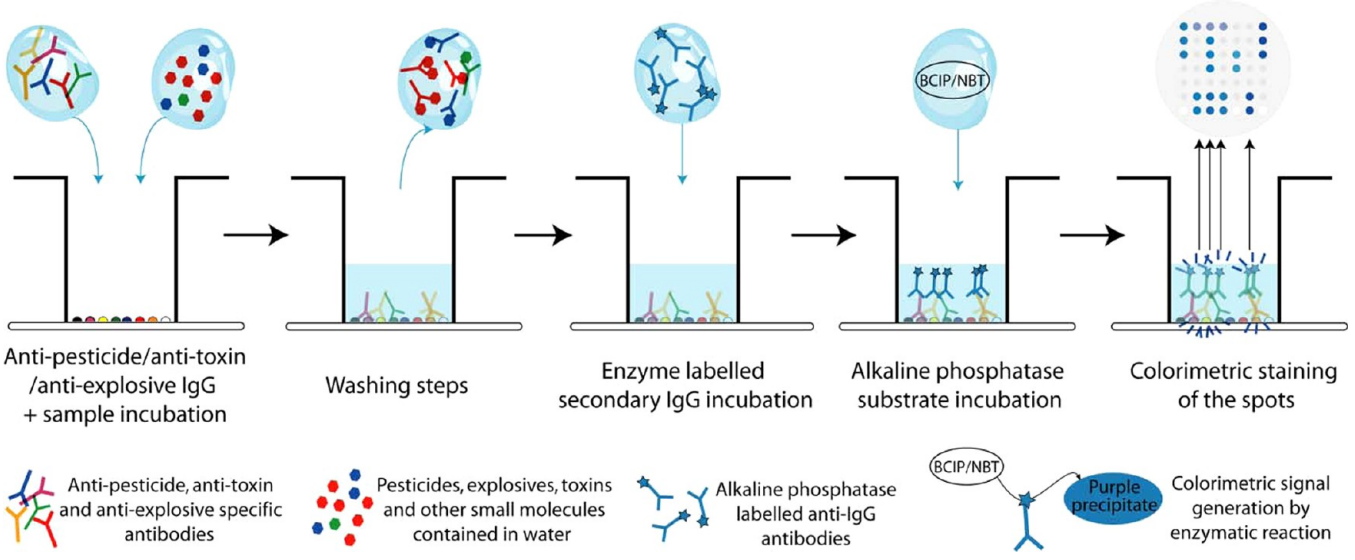

Figure 2. Workflow of the automated competitive multiplex detection of the five pollutants.

matrix was composed of 5 replicates of the chosen conjugates, one for each pollutant, triplicates of the positive control, and triplicates of the seven nonspecific interaction controls (Figure 1a). The spotted adhesive support was then directly assembled with a bottomless 96-well plate (Greiner bio-one SAS, France) thanks to its adhesive property (Figure 1b).

Automated Immunoassay Protocol. *General Protocol.* Automated immunoassays were carried out on an EVO75 robot (TECAN, France) specially equipped with a heater and a washer. The general immunoassay protocol involved the following steps: (i) the microarrays were saturated at 37 °C for 10 min with a 1:5 dilution of LowCross Buffer in phosphate buffer saline (0.1 M, pH 7.4) as a blocking agent to minimize nonspecific interaction signal. (ii) Then, the specific antipesticide, antiexplosive, and antitoxin antibodies in saturation buffer were incubated at 37 °C on the microarrays for 1 h, followed by an incubation with (iii) alkaline phosphatase labeled antimouse IgG antibodies at a concentration of 0.25 mg L^{-1} in saturation buffer. (iv) Finally, 200 μL of a BCIP/NBT ready-to-use solution were added in each well for signal generation at 37 °C for 30 min. The original color of the BCIP/NBT solution was light yellow whereas the product of the enzymatic reaction generates a purple precipitate on the spots. Between each incubation steps, and at the end of colorimetric revelation, the adhesive in each well was washed 3 times with 400 μL of phosphate buffer saline (PBS).

For colorimetric imaging and signal acquisition, the 96-well plate bottom was imaged using an ordinary flatbed scanner (HP Scanjet 3770, Hewlett-Packard) in greyscale (from 0 to 65 535 arbitrary units (au)) with a 2400 dpi resolution. Image analysis and signal quantification were performed using GenePix Pro 5.0 software (Axon Instrument). The signal intensity per spot was calculated as the median intensity for all pixels included in a circular feature defining the spot and corrected using a local background evaluation. The resulting net specific intensity of a given probe was calculated as the mean intensity of all replicates and corrected using the corresponding control.

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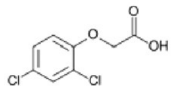
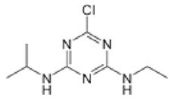
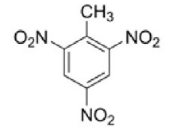
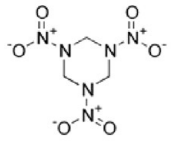
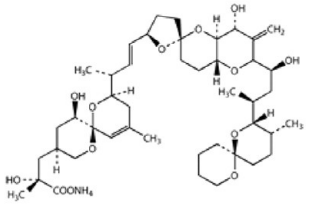
Table 1. Selected Hapten-Carrier Conjugates and Their Reactivity with the Different Antibodies

	Anti-2,4-D	*Anti-atrazine*	*Anti-TNT*	*Anti-RDX*	*Anti-OA*
BSA-2,4-D 	100.00	37.96	3.18	17.65	3.51
BSA-atrazine 	7.15	100.00	3.18	27.45	7.01
Dextran-TNT 	20.64	9.26	100.00	14.70	7.01
OVA-RDX 	9.51	74.95	9.54	100.00	7.03
BSA-OA 	9.51	12.96	6.37	2.94	100.00

Cross reaction : X < 15% ; 15%< X <30% ; X > 30%

Automated Direct Immunoassay. The automated protocol used the matrix I microarrays. The only deviation from the general protocol is the first antibodies incubation step (ii), in which concentration ranges of each specific antibody were incubated separately on the microarrays.

Automated Competitive Immunoassay. For this type of assay, the protocol was modified as presented in Figure 2 and used on matrix II microarrays. For the first antibodies incubation step (ii), a mixture of the anti-2,4-D, antiatrazine, anti-RDX, anti-TNT, and anti-OA specific antibodies at an optimized concentration of 0.1, 0.1, 1, 0.2, and 0.01 mg L^{-1}, respectively, were incubated at 37 °C for 1 h in each well, in the presence of different concentration ranges of each pollutant in order to calibrate the competitive immunoassay.

RESULTS AND DISCUSSION

Multiplex immunoassays involve multiple antibody/target couples, each one having its own optimum working conditions. Furthermore, the probability of cross-talking interferences with all the other couples evolves more than exponentially while the multiplexing number increases.[24,25] Optimizing multiplex immunoassays can then be rapidly a real experimental nightmare, and the automation/throughput of our assay is then here a real plus to rapidly test multiple assay conditions. In the field of water quality monitoring, except in the case of biological pollutions by pathogens, most of the target molecules are small organic compounds with highly similar structures, usually involving unsaturated aromatic. In the present study, the five target pollutants (Table 1) are OA, 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine (atrazine), 2,4-D, TNT, and RDX, five molecules with strong structural similarities. Since the five pollutants will have to be detected simultaneously using a competitive multiplex assay, it is then worth to foresee strong cross-reactivity issues between the different immobilized haptens and the different specific antibodies. The first step of the development of the multiplex competitive immunoassay was then the optimization of the haptens immobilization and cross-reactivity.

Microarray Preparation. In the described device, the adhesive support plays a double role: it enables one to immobilize microarrays of the hapten conjugates and to assembly these microarrays with the 96-well bottomless microtiter plates. The choice for an adhesive support was based on previous study, comparing polystyrene surfaces classically used for ELISA, porous nitrocellulose/cellulose acetate membrane, and adhesive surface.[26] The adhesive surface showed very good results in terms of colorimetric staining with the alkaline phosphatase precipitate and a very low background of nonspecific signal. Moreover, thanks to the support transparency, the colorimetric result was easily recorded. Another additional interesting feature of this adhesive material for probe immobilization is its possible integration in a complex system thanks to fast and easy assembly through its adhesive property.[27]

Probes Screening toward Selectivity. For the development of the present five-plex immunoassay, we have been screening 17 carrier-hapten conjugates toward their reactivity

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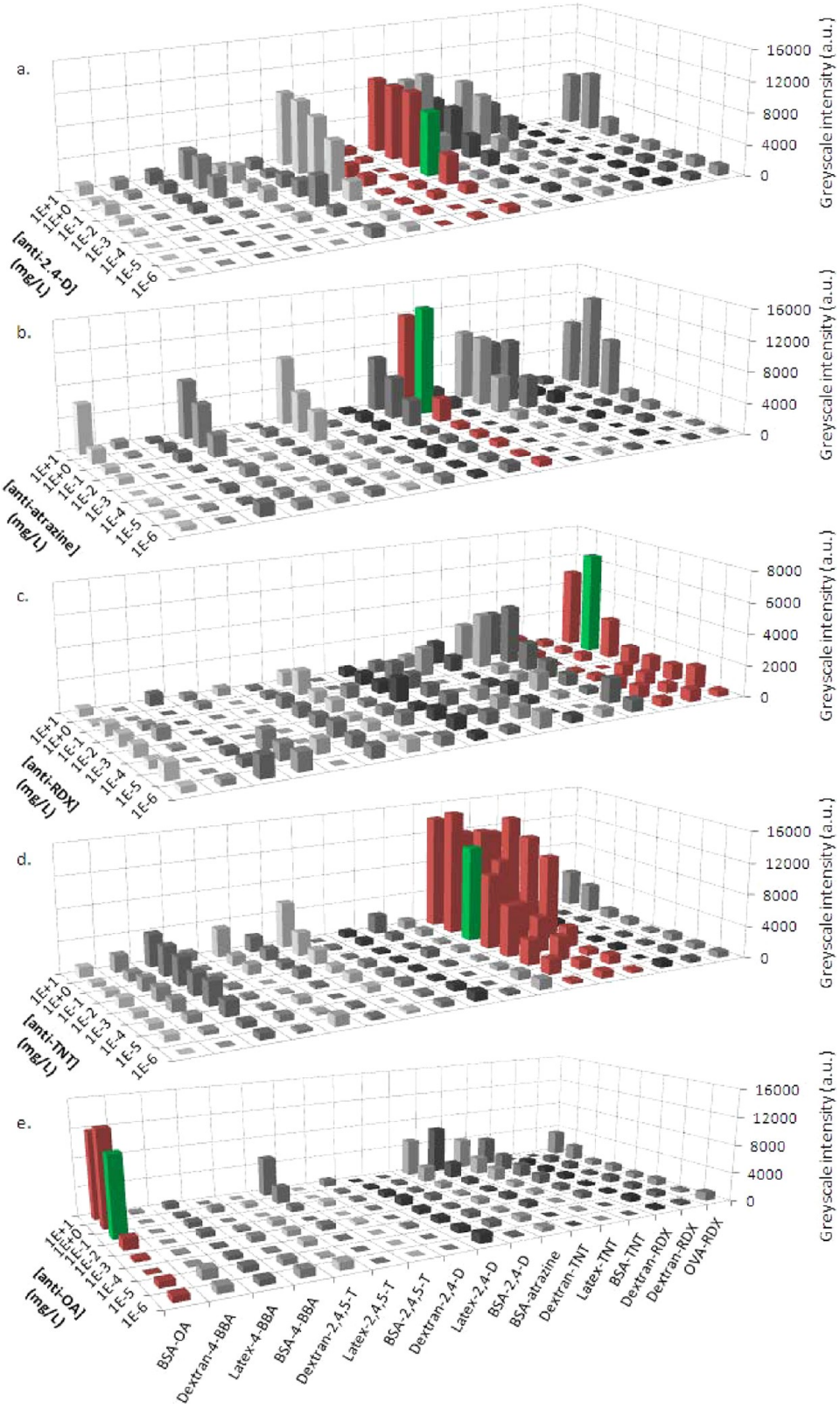

Figure 3. Cross-reactivity study between immobilized probes and antibodies. Graphical representation of the multiplex signals obtained on the different probes as a function of the different antibodies concentrations.

and cross-reactivity with specific antibodies. Different carrier molecules (BSA, OVA, dextran, and latex beads) were used in order to produce the most suitable immobilization platform for each pollutant. Indeed, a strong effect of the probe immobilization chemistry is usually observed for the competitive immunoassay of small target molecules (<500 Da). In addition to the five target molecules (atrazine, 2,4-D, RDX, TNT, and OA), two haptens (4-BBA and 2,4,5-T) were chosen for their structural

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Table 2. Cross-Reactivity of the Different Antibodies with the Different Analytes in Solution

	BSA-2,4-D	*BSA-Atz*	*OVA-RDX*	*Dextran-TNT*	*BSA-OA*
2,4-D		51.34	15.66	9.32	0.00
Atz	1.51		18.53	0.00	0.00
RDX	1.06	59.49		0.00	0.00
TNT	13.19	0.00	33.84		0.99
OA	7.21	43.98	22.46	0.00	

Cross reaction : X < 15% ; 15%< X <30% ; X > 30%

similarities and potential cross-reactivity with anti-2,4-D antibodies. 4-BBA was used as an additional control, and 2,4,5-T was expected to be valuably used for the detection of its structural analogue 2,4-D. The experiments described below were realized with the optimized conditions, determined thanks to a checkerboard titration of the antigen and antibodies simultaneously (data not shown).

As a first raw experiment, the specific reactivity and cross-reactivity of the 17 carrier-hapten conjugates were evaluated in order to select the conjugates giving the most sensitive result and the lowest cross-reactivity. Figure 3 presents the reactivity profiles of the different conjugates in the presence of increasing concentrations of specific antibodies. This representation enabled us to identify the different cross-reactivities and to select the best compromise between reactivity and specificity. As can be seen in Figure 3, from the 17 tested conjugates, 7 hapten-carriers were giving significant specific signal (BSA-OA, BSA-atrazine, BSA-2,4-D, OVA-RDX, BSA-TNT, latex-TNT, and dextran-TNT). Nevertheless, reactivity was also observed between numerous partners of the assay which force us to finely tune the antibodies concentrations and the hapten-carrier conjugates used.

The minimum signal accepted for the selection of a conjugate and of an antibody concentration was set to 6 000 au, in order to have a strong colorimetric signal for the maximum reactivity, and to be able to clearly see the decrease in competitive format. When looking closely to the profiles, the anti-2,4-D antibodies reacted only with the BSA-2,4-D conjugate and cross-reacted with all the TNT probes, with BSA-atrazine, with OVA-RDX, and with BSA-2,4,5-T but only at a concentration higher than 0.1 mg L^{-1}. The fine-tuning of the anti-2,4-D concentration (0.01 mg L^{-1}, green bar in Figure 3a) permitted then to eliminate the nonspecific signals on the RDX, TNT, and atrazine conjugates. The antiatrazine antibodies cross-reacted with all BSA conjugates, i.e., BSA-OA, BSA-2,4,5-T, BSA-2,4-D, BSA-TNT, and even the BSA-4-BBA control, but also with OVA-RDX and latex-TNT conjugates. The tuning of the antiatrazine antibodies concentration was not a satisfying solution to completely avoid cross-reaction, and the best compromise between reducing cross-reaction and keeping a strong specific signal on BSA-atrazine was then found to be an antiatrazine antibodies concentration of 1 mg L^{-1} (green bar in Figure 3b). The anti-RDX antibodies reacted only with the OVA-RDX conjugate and slightly cross-reacted with BSA-TNT and with latex-TNT. By selecting an anti-RDX antibodies concentration of 1 mg L^{-1} (green bar in Figure 3c), a maximum specific signal can be reached while lowering the cross-reaction, except on BSA-TNT and latex-TNT conjugates. The anti-TNT antibodies reacted specifically with all of the TNT-conjugates and cross-reacted weakly with all of the 2,4,5-T conjugates and with OVA-RDX. The optimization of the anti-TNT antibodies concentration (0.1 mg L^{-1}, green bar in Figure 3d) permitted one to avoid almost all cross-reaction signals while keeping a high enough specific signal. Finally, the anti-OA antibodies were shown to generate strong specific signals and weak cross-reactivity with BSA-atrazine, OVA-RDX, latex-2,4,5-T, and all of the TNT-conjugates. One more time, the selection of an anti-OA antibodies concentration of 0.1 mg L^{-1} (green bar in Figure 3e) completely circumvents cross-reactivity while keeping a high enough specific signal.

Once the different antibodies concentrations were optimized, the hapten carriers were selected according to the specific signal and cross-reactivity they generated from each specific antibody. BSA-2,4-D, BSA-atrazine, OVA-RDX, dextran-TNT, and BSA-OA were the conjugates giving the highest specific signal, and latex-TNT and BSA-TNT were discarded because of their strong reactivity with anti-RDX and antiatrazine antibodies. The 2,4,5-T conjugates, initially included in the study as an analogue useful for the detection of 2,4-D, was also abandoned because of its large cross-reactivity with other antibodies.

Table 1 presents the selected conjugates and the remaining cross-reactivity levels. As can be seen, significant cross-reactivity still occurred when using antiatrazine or anti-RDX antibodies, anticipating difficulties in the analytical treatment of the competition curves for these two pollutants.

A study of the cross-reactions of the analytes in solution was also performed. For that purpose, a cocktail of all antibodies was incubated with each analyte separately. The results of this study are summarized in Table 2. For each probe, the cross-reactivity or competitive interference of a given analyte with the different antibodies was defined as the percentage of signal decrease at the inflection point of the calibration curve (specific signal corresponding to 50%).[15]

According to the previously calculated cross-reactivity of the antibodies with the immobilized probes and the present cross-reactivity with the analytes in solution, the atrazine detection is foreseen to be hardly reliable. This issue shall in future development be specifically targeted. Methods were previously reported in order to evaluate and to avoid most of the cross-reaction issues. For example, Schuetz et al. described the selection of hapten structures for indirect immunosensors array, testing different haptens to obtain the best reactivity and selectivity with anti-TNT and anti-2,4-D antibodies.[28] Jones et al. also described the complexity of multianalyte analysis with antibodies and proposed a mathematical method to improve the experimental performance of these immunoassays and applied it to the detection of herbicides.[29]Chemometric approaches and

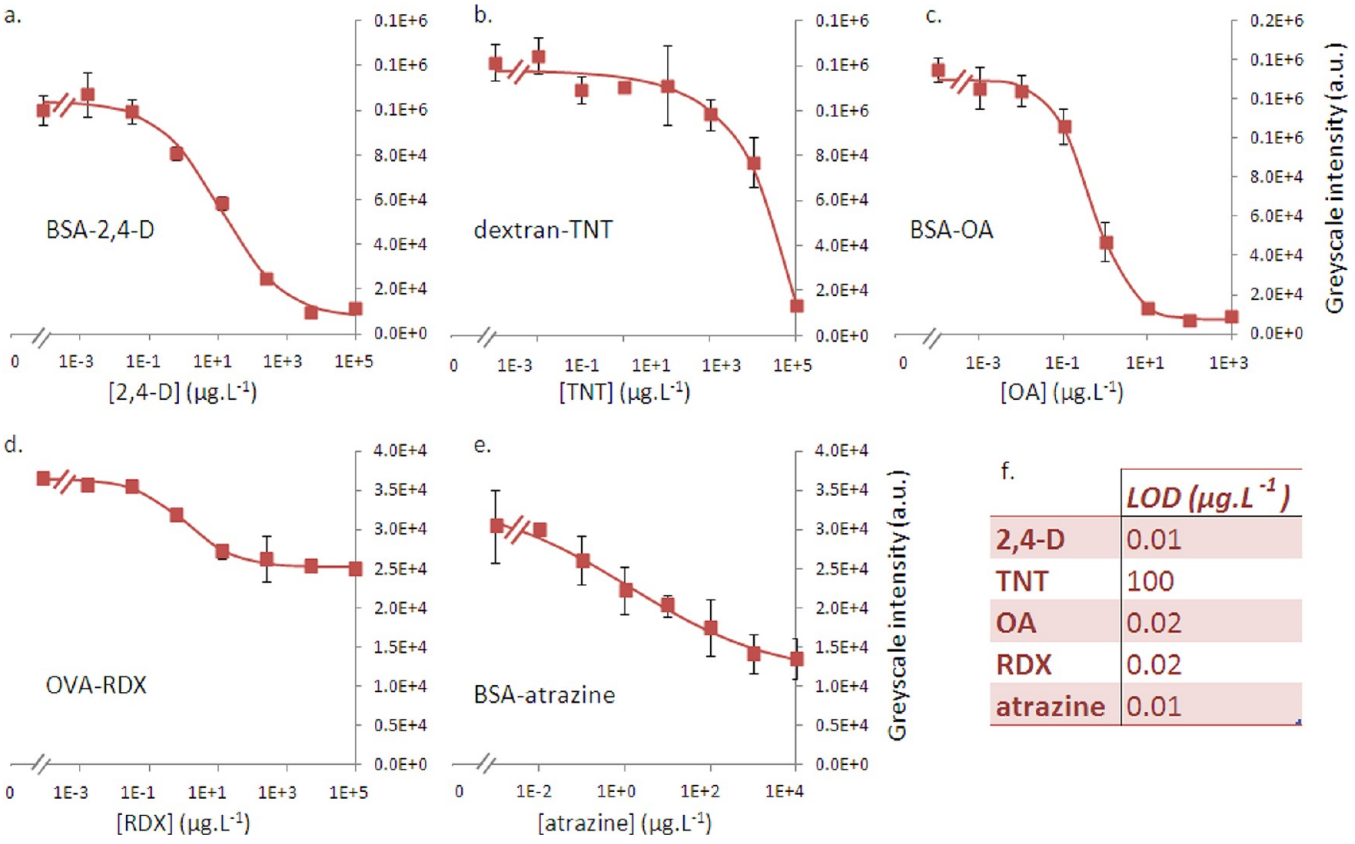

Figure 4. Calibration curves for the competitive detection of (a) 2,4-D, (b) TNT, (c) OA, (d) RDX, and (e) atrazine. Curves were fitted using a four-parameter logistic function. (f) Limits of detection of the different pollutants, calculated using 3 × mean standard deviation.

the use of a recognition pattern to classify the molecular analogue could also be an interesting tool for the determination of cross-reactivities and could improve the reliability of analyte identifications.[30]

Monoplex Calibration Curves. In order to be able later on to determine in a multiplex format the concentration of the five different pollutants, each of the five assays were calibrated independently using a cocktail of the five specific antibodies and different concentration ranges of the pollutants.

Figure 4 depicts the different calibration curves obtained for the detection of 2,4-D, TNT, OA, atrazine, and RDX. As a matter of fact, the competition occurs for all target molecules and the specific signal obtained on each probe decreases with the pollutant concentration, as expected in a competitive immunoassay. Moreover, the reproducibility of the measurement was high with a signal mean standard deviation of 9.6% (40 measurements, 5 replicates, 2 runs).

Figure 4f presents the limit of detection (LOD) for the five pollutants, determined using the four-parameter logistic fitting (Supporting Information 2). The obtained LOD (100 μg L^{-1} for TNT, 0.02 μg L^{-1} for RDX, 0.02 μg L^{-1} for OA, 0.01 μg L^{-1} for atrazine, and 0.01 μg L^{-1} for 2,4-D) were in good agreement with the EU and U.S. regulations.[3,5,6] Moreover, the sensitivity of our immunoassays is at least as good as the one obtained with previously described immunosensors using the same antibodies, except for TNT detection.[15,21,31−33]

Analyzing the atrazine and RDX calibration curves reveals an abnormal behavior at high competitor concentrations. Indeed, even if the signal levels off at high concentrations, it never reaches a zero value (or even close) and still remains at approximately 45% and 70% of the maximum signal for atrazine and RDX, respectively. These percentages have to be correlated to the highest cross-reactivity observed (Tables 1 and 2) on the different conjugates, which might generate stable signal, even at high competitor concentrations. As can be seen in Figure 3, the OVA-RDX conjugate is the only RDX conjugate that gave a signal with anti-RDX antibodies, but we also found an important cross-reactivity signal with antiatrazine and anti-2,4-D antibodies. Considering these results, the plateau obtained on the RDX competition curve can be attributed to nonspecific recognition from antiatrazine and anti-2,4-D antibodies, which are not displaced by RDX in solution. Nevertheless, we chose to keep this probe in our multiplex immunoassay because of its importance for the detection of explosive molecules, and even if the upper limit of quantification is low, our assay still can give qualitative information about the presence or absence of this molecule in the tested samples. The complexity of the involved immunochemical reactions occurring in the present system, i.e., five different antibodies having different cross-reactivity toward five different immobilized haptens, was thus here plainly evidenced.

Pollutants Detection Using the Multiplex Competitive Immunoassay. In order to evaluate the assay performances for the pollutants multiparametric detection with a semiquantitative method, spiked water samples were prepared with various concentrations of the different targets. The composition of the samples is given in Table 3 together with the recovery values (R) calculated using the concentrations determined thanks to the equations of the four-parameter fitting curves determined previously (Supporting Information 2).

As can be seen, 82.5% of the spiked concentrations were successfully determined using the present multiplex competitive

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Table 3. Recovery Values Obtained during the Multiplexed Detection of Various Spiked Water Samples

	Pollutant	Recovery R = $[pollutant]_{calculated}/[pollutant]_{real}$				
Sample #	Composition [pollutant] ($mg.L^{-1}$)	RDX	TNT	atrazine	2,4-D	OA
1	RDX: 0 TNT: 0.5 Atrazine: 0 2,4-D: 0.005 OA: 0		73 %		124 %	
2	RDX: 0.05 TNT: 0 Atrazine: 0 2,4-D: 0.05 OA: 0	82 %			107 %	
3	RDX: 0 TNT: 0 Atrazine: 0.005 2,4-D: 0.5 OA: 0			81 %	103 %	
4	RDX: 0 TNT: 0 Atrazine: 0 2,4-D: 0 OA: 0.1			~LOD		ULQ[a]
5	RDX: 0 TNT: 1 Atrazine: 0 2,4-D: 0 OA: 0	~LOD	90 %			
6	RDX: 0.01 TNT: 0 Atrazine: 0 2,4-D: 0 OA: 0	109 %				
7	RDX: 0 TNT: 0 Atrazine: 0.01 2,4-D: 0 OA: 0			96 %		
8	RDX: 0 TNT: 0 Atrazine: 0 2,4-D: 0.1 OA: 0				126 %	
9	RDX: 0 TNT: 0.0005 Atrazine: 0 2,4-D: 0 OA: 0.05		LOD			ULQ[a]
10	RDX: 0.0005 TNT: 0 Atrazine: 0 2,4-D: 0 OA: 0.005	129 %				108%
11	RDX: 0.5 TNT: 0.005 Atrazine: 0 2,4-D: 0 OA: 0	101 %	LOD			
12	RDX: 0 TNT: 0 Atrazine: 0.0005 2,4-D: 0 OA: 0.0005			LOD		115 %
13	RDX: 0 TNT: 0.05 Atrazine: 0.005 2,4-D: 0 OA: 0		LOD	126 %		
14	RDX: 0.005 TNT: 0 Atrazine: 0.05 2,4-D: 0 OA: 0	130 %		121 %		
15	RDX: 0 TNT: 0 Atrazine: 0 2,4-D: 0.0005 OA: 0.00005				LOD	LOD
16	RDX: 0 TNT: 0 Atrazine: 0 2,4-D: 0 OA: 0					

[a] ULQ : real value are higher than the upper limit of quantification.

Overestimated:	3.75 %
Significantly different:	13.75 %
Successfully determined:	82.5 %

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assay (green boxes in Table 3). Matching concentrations were defined as significantly close values (i.e., 85% ≤ R ≤ 115%). Moreover, every spike concentration known to be lower than the LOD of a particular target were considered to be successfully determined when a zero value was calculated.

Besides, 13.75% of the calculated concentrations were found to be significantly different but not incoherent (70% ≤ R < 85% and 115% < R ≤ 130%) from the spiking values (orange boxes in Table 3). Then, 3.75% of the concentrations were overestimated (R < 70% and R > 130%), leading to false positive results (red boxes in Table 3). Interestingly, atrazine was the only target to generate a false positive but only in the presence of RDX, 2,4-D, or TNT, evidencing one more time the impact of the cross-reactivity of the antiatrazine with the other haptens and analytes (Tables 1 and 2) on the analytical results.

CONCLUSIONS

In this proof of concept study, we demonstrated the applicability of an adhesive-based microarray in a 96-well plate format for the multiparametric detection of three different types of small molecule water pollutants.

The optimization of the immobilized probes, of the protocol conditions, and of the cross-reactions was highly facilitated thanks to the fully automated protocol possible only because of the standard architecture of the tool (96-well plate). Moreover, with less than 3 h assay duration for 96 samples and whatever the number of parameter determined, our system was shown to be compatible with high-throughput requirements.

Nevertheless, as expected when dealing with multiplex immunoassays, cross-reactivity of the assay components was a real bottleneck. The optimization of the assay conditions and hapten-carrier compositions helped us partially overcome this problem, but interferences still remain which are generating false positive results.

In previous reports, the Pla-Roca group proposed an interesting approach based on antibody colocalization in order to surmount the major issue of cross-reaction in multiparametric immunoassays.[25] With their system, the specific antibodies did not interact with each other or with the other probes and the cross-reactions are avoided. Another alternative, possible only when using the present adhesive microarray, shall be to combine the multiplexing properties of the microarray with the ultrahigh throughput of a 384 or 1536-well plate.[34] Separating the cross-reacting species in different microwells shall then lead to more accurate analytical results while keeping the multiplex analysis possible. Indeed, up to 100 spots per well can be obtained in 384 format and 25 in a 1536 format. This is clearly an interesting avenue for future developments of the present water monitoring system.

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.

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Notes

The authors declare no competing financial interest.

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