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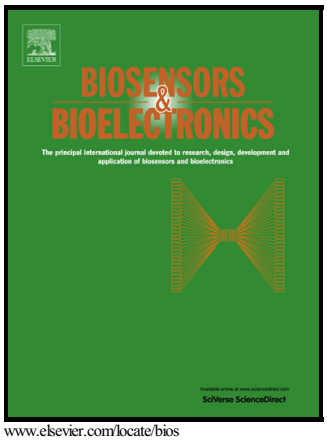

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DEVELOPMENT OF A HIGHLY SENSITIVE NONCOMPETITIVE ELECTROCHEMICAL IMMUNOSENSOR FOR THE DETECTION OF ATRAZINE BY PHAGE ANTI-IMMUNOCOMPLEX ASSAY

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Abstract

The development of immunosensors for the detection of small molecules is of great interest because of their simplicity, high sensitivity and extended analytical range. Due to their size, small compounds cannot be simultaneously recognized by two antibodies impeding their detection by noncompetitive two-site immunoassays, which are superior to competitive ones in terms of sensitivity, kinetics, and working range. In this work, we combine the

advantages of magneto-electrochemical immunosensors with the improved sensitivity and direct proportional signal of noncompetitive immunoassays to develop a new Phage Anti-Immunocomplex Electrochemical Immunosensor (PhAIEI) for the detection of the herbicide atrazine. The noncompetitive assay is based on the use of recombinant M13 phage particles bearing a peptide that specifically recognizes the immunocomplex of atrazine with an anti-atrazine monoclonal antibody. The PhAIEI performed with a limit of detection (LOD) of 0.2 pg mL^{-1}, which is 200-fold better than the LOD obtained using the same antibody in an optimized conventional competitive ELISA, with a large increase in working range. The developed PhAIEI was successfully used to assay undiluted river water samples with no pretreatment and excellent recoveries. Apart from the first demonstration of the benefits of integrating phage anti-immunocomplex particles into electrochemical immunosensors, the extremely low and environmentally relevant detection limits of atrazine attained with the PhAIEIS may have direct applicability to fast and sensitive detection of this herbicide in the environment.

Keywords: Anti-immunocomplex peptide, Immunosensor, Atrazine, Chronoamperometry

1. Introduction

The enzyme immunoassay with electrochemical detection, which combines the selectivity of antibodies with the sensitivity of electrochemical techniques, has become a powerful tool for the analysis of clinical, environmental, food and commodity samples, showing a high sensitivity and

extended analytical range (Han et al. 2013; Lou et al. 2013). Electrochemical techniques are particularly suited for rapid and direct detection of antibody–antigen interactions, which adds to the advantageous high specificity of antibodies making possible to eliminate or simplify the sample cleanup, making the assay rapid and cost-effective.

The need for simple, rapid, reliable and economic detection of small molecules such as pesticides, mycotoxins, drugs and hormones in environmental and biomedical analysis is growing. The central component of these assays is the analyte-antibody reaction. If the analyte is a macromolecule, typically it is first captured by a primary antibody and then detected with a secondary antibody coupled to a tracer. This two-site format allows the use of excess concentrations of reacting antibodies, which taking into consideration the law of mass action, promotes the formation of the trivalent immunocomplex, even in the presence of trace amounts of the analyte (high assay sensitivity). In the case of immunoassays for small molecules, which usually have a single epitope, after the antigen-antibody interaction takes place up to 85% of the antigen surface is buried in the paratope of the antibody (Lamminmaki and Kankare 2001; Monnet et al. 2002) precluding a possible interaction with a second antibody (Jackson and Ekins, 1986). This rules out the use of two-site assays, and for this reason, small molecules are detected in an indirect way (competitive immunoassays) in which the analyte competes for binding to the detecting antibody with a tracer compound. In this format, the presence of the analyte is registered as a loss in signal (Deshpande, 1996) and limiting amounts of both antibody and tracer have to be used to obtain measurable inhibition with

trace concentrations of the analyte. The use of limiting concentration of reactants is unfavorable for the formation of the immunocomplex and hence, the sensitivity obtained with this format is inferior to that achieved with noncompetitive ones (Jackson and Ekins, 1986).

Noncompetitive immunoassays for the detection of small molecules can be developed by using short peptide loops that specifically react with the exposed region of the hapten and the conformational changes caused by its binding (Gonzalez-Techera et al. 2007b). These peptides are isolated from phage display peptide libraries that contain a vast repertoire of peptidic sequences (typically around $1x10^{9-10}$ individual clones) expressed on the surface of the filamentous phage M13 of the fd family (Scott and Smith, 1990). Phage libraries are enriched for specific clones by repetitive rounds of affinity selection (biopanning), which includes binding to the desired selector molecule, washing and elution, reinfection of bacteria, and growth to amplify the selected phages. Once selected, the phage can be easily produced in large amounts in an inexpensive way, and perform as robust reagent, that can withstand harsh conditions of pH 2-12, up to 70°C and denaturants. We have previously shown that phages bearing anti-immunocomplex peptides can be isolated from libraries and used as recognition agents for small molecules-antibodies complexes, in an ELISA format called phage anti-immunocomplex assay (PHAIA) (Gonzalez-Techera et al. 2007a; Gonzalez-Techera et al. 2007b; Rossotti et al. 2010). This strategy can be used to “convert” almost any competitive ELISA into a noncompetitive one, which usually performs with at least a 10-fold improvement in sensitivity. However, thus far, the potential of

PHAIA has not been explored in other formats, such as electrochemical immunosensors, despite the fact that the phage particles have shown advantageous properties for such applications (Arévalo et al. 2012).

On the other hand, for the immunosensors development the immobilization of the antibodies onto the solid surface is a key step that determines the stability, reproducibility and sensibility of the measured signal (Cosnier, 1999). The use of magnetics beads (MBs) coated covalently with Protein G confers specific binding and orientation of the captured antibodies (Margni, 1996; Liang et al. 2008), with additional advantages of easy handling and high reaction kinetics (Lin et al. 2007; Font et al. 2008).

In this work we report the use of noncompetitive phage anti-immunocomplex assay, in combination with a magneto-electrochemical immunosensor, for the detection and quantification of atrazine. The original immunoassay, which already exhibited a very high sensitivity in a competitive chemical hapten based format (LOD = 0.18 ng mL^{-1}) (Giersch et al. 1993) showed an improvement in sensitivity of approximately 10-fold when performed as PHAIA with the same antibody in a colorimetric ELISA (Gonzalez-Techera et al. 2007b). Atrazine (MW = 215 g mol^{-1}) is a herbicide of the "triazines" family, that is usually applied to soil as pre-emergence after crop planting (Park et al. 2014) with systemic and residual action. Atrazine is one of the most heavily used herbicides worldwide and it has been identified as an endocrine disrupting chemical and a potential carcinogen. For these reasons the development of

highly sensitive detection and quantification techniques to assess water quality are needed (Tao and Tang, 2004).

Using the atrazine specific monoclonal antibody (MoAb K4e7) (Giersch et al. 1993), and phage particles bearing a short peptide loop (13A clone) that specifically recognizes MoAb K4e7-atrazine immunocomplexes, a Phage Anti-Immunocomplex Electrochemical Immunosensor (PhAIEI) was developed. The PhAIEI uses MBs functionalized with protein G as solid phase for the MoAb K4e7-atrazine-phage reaction, an anti-M13 monoclonal antibody coupled to horseradish peroxidase (HRP) for phage detection. Hydrogen peroxide and pyrocatechol were used as enzymatic substrate and redox mediator, respectively. The benzoquinone produced by the enzymatic reaction was then detected on a carbon screen printed-electrode (CSPE) by chronoamperometry (CA). We found a remarkable increase in sensitivity with regard to the standard noncompetitive immunoassay ELISA with a significant time reduction of the assay.

2. Material and methods

2.1. Chemicals, antibodies and other reagents

All reagents used were of analytical grade. Atrazine was a gift from Dr. Shirley Gee, and anti-atrazine mouse monoclonal antibody (MoAb) K4e7 was a gift from Dr. T. Giersch (Giersch et al. 1993). Anti-M13 phage monoclonal antibody conjugated to horseradish peroxidase (α-M13-HRP) was purchased

from Pharmacia (Uppsala). Pyrocatechol (H_2Q), 3,3-,5,5-tetramethylbenzidine (TMB) and bovine serum albumin (BSA) were obtained from Sigma–Aldrich. Dimethylsulfoxide (DMSO) and H_2O (HPLC grade) were purchased from Sintorgan. The following buffer solutions were prepared from their salts (Merck, p.a.): 1 x 10^{-2} M KH_2PO_4 + Na_2HPO_4, 0.137 M NaCl and 2.70 x 10^{-3} M KCl, pH 7.0 (phosphate buffer solution, PBS); 5 x 10^{-2} M $Na_3C_3H_5O(COO)_3$ + 5 x 10^{-2} M Na_2HPO_4, pH 5.00 (citrate buffer solution, CBS); 5 x 10^{-2} M $Na_3C_4H_5O_4$ + 5 x 10^{-2} M $NaC_2H_3O_2$ (acetate buffer solution), pH 5.5 and PBS containing 0.05% Tween 20 (PBST). H_2O_2, $MgSO_4$ and H_2SO_4 were Merck p.a. Water samples were obtained by collecting surface water from the Rio Cuarto river, Rio Cuarto, Argentina, and were spiked with different amounts of atrazine.

2.2. Materials and apparatus

The CSPE based on a disk shape working (1.5 mm of dia.) and ring shape counter electrodes of carbon and a pseudo-reference electrode of silver were purchased from Palm Sens. Magnetics beads modified with protein G (MBs) were Dinabeads® (Invitrogen). These MBs (2.8 μm diameter) have a high binding capacity, approximately 8 μg human IgG per mg of MBs. Before use, the MBs were washed with PBS and loaded with saturating amounts of MoAb K4e7 as described below. Nunc Maxisorp plates (96 well) were purchased from Nunc. A neodymium high field magnet was used for separations. Chronoamperometry (CA) measurements were performed with an AutoLab PGSTAT30 potentiostat, run with the GPES (version 4.9) electrochemical analysis software from Eco-chemie, Utrecht, The Netherlands.

Colorimetric measurements were performed with a Multiskan EX ELISA reader. The samples with MBs were mixed with a Vortemixer Speed Knob vortex. All steps of immunoassay were performed at 37 °C using a stove NEO LINE thermostat, Argentina.

2.3. Preparation of stabilized phage suspensions

The amplified phage clone 13 A which bears the anti-immunocomplex peptide sequence CTPVRWFDMC, specific for the MoAb K4e7-atrazine complex was obtained as described previously (Gonzalez-Techera et al. 2007b). After two steps of precipitation with PEG-NaCl, the phage particles were suspended in 1:50 volume of the original culture volume in PBS, which was supplemented with the Complete Protease Inhibitor Cocktail of Roche Diagnostics and 0.05% sodium azide. The preparations were filtered through a 0.22 μm filter and stored in aliquots at 4 °C. The phage suspension was titrated in ARI 292 *E.coli* cells, and the final phage concentration was 1 x 10^{12} phage particles mL^{-1}.

2.4. Checkerboard titrations for the development of sensitive standard microtiter plate-based ELISA for atrazine detection

Optimization of MoAb K4e7 and phage particles concentrations were performed on a 96-well high binding microtiter plate. ELISA plates were coated with 50 μL of MoAb K4e7 in concentrations of 10, 5 and 2.5 μg mL^{-1} (two rows for each concentration). After incubating for 1 h at room temperature the wells

were washed with PBS and blocked with 1% (w/v) BSA in PBS for 1 h. Later, the wells were washed with PBST. Then, 50 μL of a solution of atrazine (100 ng mL^{-1}) was added to all wells and incubated for 1 h at room temperature. The wells were washed with PBST and two-fold serial dilutions of phage particles, starting at a 1:10 dilution (1 x 10^{11} phage particles mL^{-1}), then added to columns 1–11 and incubated for 1 h at room temperature. Wells were washed with PBST and 50 μL of a 1:5000 dilution of a MoAb α-M13-HRP in PBST was added to each well. After 1 h, the plates were washed with PBST and 50 μL of the HRP substrate (0.4 mL of a 6 mg mL^{-1} DMSO solution of TMB and 0.1 mL of 1% H_2O_2 in water in a total of 25 mL of 0.1 M citrate-acetate buffer, pH 5.50) was dispensed into each well. The enzymatic reaction was stopped after 15 min by the addition of 50 μL of 2 M H_2SO_4, and the absorbance was measured at 450 nm (corrected at 600 nm).

2.5. Noncompetitive ELISA for atrazine determination

50 μL per well of serial dilutions of atrazine standard in PBST were dispensed into microtiter plates coated with MoAb K4e7 and blocked with 1% (w/v) BSA in PBS. The wells were incubated for 1 h at room temperature. Later, the wells were washed with PBS, followed by addition of 50 μL per well of an adequate dilution of the stabilized phage suspension. The MoAb k4e7 concentration used for coating and the proper dilution of the phage suspension had been previously optimized by checkerboard experiments, as described above. After 1 h of incubation at room temperature, the wells were washed with PBST and 50 μL of a 1:5000 dilution of MoAb α-M13-HRP in PBST was added

to each well. After 1 h, the plates were washed with PBS. The development of color was obtained as previously described.

2.6. Optimization of the components of the magneto-electrochemical immunosensor

The concentrations of MoAb K4e7, phage particles, H_2O_2 and H_2Q and amount of MBs for the PhAIEI had to be optimized. The MBs were incubated with 50 μL of a solution of MoAb K4e7, at final concentrations of 2.5, 5 and 10 μg mL^{-1}. After incubating and stirring at 200 rpm for 30 min at 37 °C, the MoAb K4e7–MBs complexes were washed with PBS (a high magnetic field was used for separating the MBs from the solution). Then, a solution of 3% mouse serum (to prevent protein G capture of the generating signal antibody, the MoAb α-M13-HRP) was added, and incubated with stirring to 200 rpm for 30 min at 37 °C. The MBs complexes were precipitated and washed with PBST. Later, 50 μL of a solution of atrazine at 100 ng mL^{-1} was added to MBs and incubated with stirring for 30 min at 37°C. A magnetic separation and washing were then performed on MBs complexes with PBS and two-fold serial dilutions of phage particles, starting at a 1:10 dilution (1 x 10^{11} phage particles mL^{-1}) an ending in 1:640 dilution (1.5 x 10^{8} phage particles mL^{-1}), were added to the immune MBs and stirring at 200 rpm at 37 °C for 30 min. The MBs complexes were precipitated and washed with PBS and 50 μL of a 1:5000 dilution of a MoAb α-M13-HRP in PBS was added, and the solution was stirred at 200 rpm at 37 °C for 30 min. The MBs complexes were washed and 25 μL of a solution of H_2Q in CBS was added and transferred to CSPE. Chronoamperometry was performed

as described below. The concentrations studied for the substrate and co-substrate were varied from 1 x 10^{-3} mM to 5 mM.

2.7. Noncompetitive phage anti-immunocomplex electrochemical immunosensor for atrazine quantitation

A heterogeneous noncompetitive immunoassay was used for the development of the atrazine PhAIEI. Briefly, suspensions of 1 μL of MBs were transferred to Eppendorf tubes and washed three times with PBS, to remove the NaN_3 used as preservative. Then, 50 μL of MoAb K4e7 (10 μg mL^{-1} in PBS) was added and stirred at 200 rpm at 37 °C for 30 min to obtain MoAb K4e7–MBs complexes. After incubation, a high magnetic field was used for separating the MBs loaded with the antibody. The MBs were washed with PBS and were incubated with 50 μL of 3% (v/v) mouse serum in PBS for 30 min at 37 °C stirring at 200 rpm, in order to prevent direct binding of MoAb α-M13-HRP to the remaining protein G binding sites. The MBs were then washed as described above, and re-suspended in 50 μL of the atrazine standards or water samples, stirring to 200 rpm at 37 °C for 30 min. MBs were then magnetically separated, the supernatant was removed and MBs were washed with PBS. The MBs were re-suspended in 50 μL of phage particles of clone 13A, in a dilution factor of 1:40 (2.5 x 10^{10} particles mL^{-1}) in PBS, stirring to 200 rpm at 37 °C for 30 min. MBs were magnetically separated, the supernatant was removed and MBs were washed with PBS to remove non-specific bound phage particles. 50 μL of MoAb α-M13-HRP (dilution of 1:5000 in PBS) were added to MBs and stirred at 200 rpm at 37 °C for 30 min. The MBs were washed with PBS and deposited

with the magnet. Then, the MBs were resuspended in 25 μL of a solution of H_2Q 1.2 x 10^{-3} M, in CBS. The suspension was carried out on SPCE surface, and a potential of −0.2 V was applied. After 100 seconds (when the current value was constant), 5 μL of a 6 x 10^{-3} M H_2O_2 solution was added to the SPCE surface (both final concentrations were 1 x 10^{-3} M). After 60 seconds the steady state current (*I*) was achieved. In the presence of H_2O_2, HRP catalyzes the oxidation of H_2Q to Q (Ruan and Li, 2001) and its back electrochemical reduction to H_2Q can be detected on the CSPE through chronoamperometry measuring the steady state current. The schematic representation of the PhAIEI is shown in Fig. 1. The steady state current obtained is proportional to the amount of HRP which in turn is proportional to the amount of atrazine.

3. Results and discussion

3.1. Microtiter plate based noncompetitive ELISA for atrazine determination

Checkerboard titration analysis was used to optimize both the amount of coating antibody and the final 13A clone phage concentration in the presence of a fixed amount of atrazine and in the absence of it, in order to set up the best conditions for noncompetitive ELISA colorimetric assay, as described in section 2.4. A coating concentration of MoAb K4e7 of 10 μg mL^{-1} produced the highest signal in combination with a 1:160 dilution (which corresponds to 6.25 x 10^9 phage particles mL^{-1}) of stabilized stock phage suspension with a negligible signal in the absence of atrazine (Fig. S1, Supplementary material). Once the amount of antibody and final phage concentration were fixed, it was possible to

develop a noncompetitive colorimetric ELISA to measure the analyte-bound sites. The curve obtained is shown in Fig. 2, and is represented as binding percentage (B/B_0 = absorbance value/absorbance value obtained for the saturation of atrazine) *vs* $c^*_{atrazine}$ (atrazine concentration) and was fitted to a four-parameter logistic equation (Rodbard, 1974):

$$y = \quad + \left[\qquad \frac{A -}{} \right] \qquad (1)$$

where A and D are the maximum and minimum B/B_0 values, respectively, SC_{50} is the concentration of atrazine which produces 50 % of signal saturation and Hill Slope is the slope at the midpoint of the sigmoid curve. The limit of detection (LOD) of the assay was calculated as the concentration of atrazine causing an increase in signal equal to three times the standard deviation of the blank (ACS, 1980). The LOD was 0.040 ng mL^{-1} and the SC_{50} = 0.055 ng mL^{-1}. The calibration curve showed a linear range from 0.01 to 0.1 ng mL^{-1}. These values are similar to those previously reported by us (Gonzalez-Techera et al. 2007b).

3.2. Noncompetitive magneto-electrochemical immunosensor for atrazine determination

Different parameters were studied to optimize the performance of the PhAIEI, including the concentration of MoAb K4e7, concentration of phage particles, the amount of MBs, volume and concentration of redox mediator and enzymatic substrate solutions and nonspecific adsorptions. It is know that the oxidation of H_2Q to Q and the reduction of Q back to H_2Q, is a quasi-reversible two-electron redox process (Forzani et al. 1997). Using CSPE this redox

process occurs with peak potential of 0.330 V for the oxidation of H_2Q and 0.138 V for the reduction of Q, when 1 x 10^{-3} M H_2Q in CBS is used. H_2O_2 was not reduced in the potential range studied (Fig S2, Supplementary material). For the development of the PhAIEI assay, the potential chosen for all the experiments of Q reduction by chronoamperometry was –0.2 V.

3.2.1. Optimization of the phage particles and MoAb K4e7 final concentrations for the PhAIEI assay development

As previously observed in the noncompetitive microtiter plate based ELISA, the best sensitivity is attained when the assay is performed with the highest concentration of phage particles and coating antibody that keeps a low background readout in the absence of analyte (Fig S1, Supplementary material). This was explored by varying the dilution factor of the phage particles between 1:10 and 1:1280, and capturing MoAb K4e7 from solutions with concentrations ranging from 2.5 to 10 μg mL^{-1} (Section 2.6). The maximum amount of both phage particles bearing the anti-immunocomplex peptide 13A clone and MoAb K4e7 that could be used without generating a signal in the absence of atrazine was achieved at a 1:40 dilution (2.5 x 10^{10} phage particles mL^{-1}) of the stabilized stock phage suspension and 10 μg mL^{-1} of antibody, Fig. 3.a. Also, a concentration of 2.5 x 10^{10} phage particles mL^{-1} in combination with MBs coated with 10 μg mL^{-1} of MoAb K4e7 resulted in much clearer differential signals than the ones obtained with MBs coated with 2.5 and 5 μg mL^{-1} when atrazine was present in a wide range of concentrations (1, 100 and 10 x 10^3 pg mL^{-1}), as shown in chronoamperograms of Fig. 3.b. Therefore, a dilution factor

of phage particles of 1:40 (corresponding to 2.5 x 10^{10} phage particles mL^{-1}) and a concentration of K4e7 MoAb of 10 μg mL^{-1} were chosen for performing the following PhAIEI assays in optimal conditions.

3.2.2. Optimization of the amount of magnetic beads used in the PhAIEI assay

The amount of MBs was varied from 7.5 to 60 μg (0.25 to 2 μL of MBs solution) in combination with MoAb K4e7 at a concentration of 10 μg mL^{-1}, a dilution factor of phage particles of 1:40 (corresponding to 2.5 x 10^{10} phage particles mL^{-1}) and atrazine at a final concentration of 100 ng mL^{-1} (Fig. 4). The increase in the amount of MBs produced an increase in the current values reaching its maximum at 30 μg of MBs, which was thus used for the development of the PhAIEI. The incubation time was also studied for each step of the immunoassay, and the current value increased up to 30 min, then it remained basically constant (data not shown). This value was used for each incubation step.

3.2.3. Study of H_2Q and H_2O_2 concentration and reaction volumes

Different concentrations of H_2Q and H_2O_2 were explored (Section 2.6). Concentrations of H_2Q were varied from 1 x 10^{-5} M to 5 x 10^{-3} M with a H_2O_2 solution of 1 x 10^{-3} M. An increase of current was observed with increasing H_2Q concentration, Q was continuously produced and a steady state current was difficult to obtain. On the other hand, concentrations of H_2O_2 were varied from 1 x 10^{-5} M to 5 x 10^{-3} M with a H_2Q solution of 1 x 10^{-3} M (Fig. S3, Supplementary

material). Again, an increase of current was obtained with increasing H_2O_2 concentrations, but poor reproducibility was obtained for concentrations higher than 1 x 10^{-3} M of H_2O_2, and then, the concentrations of H_2Q and H_2O_2 chosen were 1 x 10^{-3} M in both cases. Additionally, the reaction volume added to CSPE was also studied. The current value increases with volume reduction, due to an increment of concentration of enzymatically produced Q, and in this way, a 30 μL reaction volume was chosen.

3.2.4. Calibration curve for atrazine using PhAIEI assay.

A dose-response titration for atrazine was carried out in the concentration range 1 x 10^{-3} to 1x 10^4 pg mL^{-1} (Fig. 5) using the PhAIEI. The calibration curve was plotted as binding percentage (B/B_0 x 100) *vs* $c^*_{atrazine}$, were B is the steady state current (*I*) obtained, and B_0 is the steady state current (I_0) obtained with analyte saturation. The calibration curve was fitted using Equation (1). The sensitivity (SC_{50}) and LOD were 1.3 pg mL^{-1} and 0.2 pg mL^{-1}, respectively, with a Hill Slope of 0.75 ± 0.12. This Hill Slope value agrees with the linear range of concentrations (0.2 pg mL^{-1} to 20 pg mL^{-1}). The SC_{50} and LOD were 50-fold and 200-fold better than those obtained for the colorimetric noncompetitive ELISA microtiter-plate-based immunoassay performed with the same antibody and anti-immunocomplex peptide borne phage. Various analytical electrochemical immunoassays have been described in the literature related to the determination of atrazine, with LODs that span a range from 6 pg mL^{-1} to 15 ng mL^{-1} (Zacco et al. 2006; Valera et al. 2007; Ionescu et al. 2010; Campanella et al. 2011; Giannetto et al. 2014; Liu et al. 2014). The sensitivity of the PHAIEI

presented here represents a 30-fold improvement in sensitivity compared to the most sensitive electrochemical based immunoassay reported for atrazine, to date (Zacco et al. 2006). The accuracy and reproducibility of the PhAIEI was checked using standard solutions of atrazine of 100 and 1 pg mL^{-1}. The intra-assay parameters were tested by performing three consecutive measurements of the same sample. These series of measurements were repeated for three consecutive days to estimate the inter-assay values. As summarized in Table 1, the PhAIEI assay for atrazine showed a very good accuracy and reproducibility. Cross-reactivity with related compounds was not performed in this work. However, this was previously done using the same MoAb and anti-immunocomplex peptide in our initial work describing PHAIA (Gonzalez-Techera et al. 2007b). In this work, cross-reactivity studies with: simazine, propazine, cyanazine, ametryn, simetryn, prometryn, terbutryn and propazine were performed. Only propazine exhibited 116% cross-reactivity with the chemical hapten and 144% with peptide clone 13A). We expect that the cross-reactivity with triazines compounds to remain the same as those previously obtained in standard colorimetric microtiter noncompetitive ELISA.

3.3. Atrazine determination in river water samples using the PhAIEI assay

The performance of the PhAIEI assay with real samples was tested using undiluted river water samples obtained from the Río Cuarto River, Córdoba, Argentina. Preliminary analysis of non-spiked river samples showed zero readings when analyzed by ELISA or PhAIEI (data not shown). Then, atrazine

was spiked with 100 and 1 pg mL^{-1} and each sample was analyzed in triplicates. Excellent recoveries of 99 ± 6.8 % and 96 ± 5.7 % were obtained for both samples, respectively. These results demonstrate that the PhAIEI has a high potential for ultra-sensitive water analysis even when undiluted samples are used.

4. Conclusions

We recently described the use of phage particles for the development of ultra-sensitive competitive electrochemical sensor for the herbicide molinate (187 g mol^{-1}), using analytes peptidomimetics (Arévalo et al. 2012). The filamentous nature of the fd phage proved to be of great importance for electrochemical detection. Over 2500 copies of the pVIII phage coat protein are expressed on the virion particle; since pVIII is the target epitope of MoAb α-M13-HRP, a large number of HRP molecules are bound to each phage providing a strong activity for pycocatechol oxidation in hydrogen peroxide presence. In this new development, this advantageous feature of the phage was combined with the improved sensitivity afforded by the use of anti-immunocomplex peptides to generate an ultrasensitive immunosensor for non-competitive electrochemical detection of atrazine, one of the most heavily used herbicides worldwide. As a result, the LOD of the PhAIEI (0.2 pg mL^{-1}) was 900 fold better than the LOD (180 pg mL^{-1}) obtained with the same antibody in a conventional chemical hapten format (Giersch et al. 1993). The assay adaptation into the electrochemical format also improved with regard to PHAIA providing a 200 fold improve in sensitivity (LOD = 40 pg mL^{-1}) with a 10-fold

extended linear dose-response range. The PhAIEI showed excellent results for atrazine determination in untreated river samples. Given that anti-immunocomplex peptides can be isolated in a straightforward manner from phage display libraries resulting in noncompetitive assays with improved sensitivity, the development of PhAIEI assays stands as a salient option to boost the sensitivity of current electrochemical competitive immunosensors for the detection of small molecules.

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Figure Captions:

Figure 1. Schematic representation of the phage anti-immunocomplex electrochemical immunosensor (PhAIEI) for atrazine. Left panel: immunoassay components. Right panel: schematic representation of the electrochemical reduction involving the enzymatic process among pyrocatechol (H_2Q), benzoquinone (Q), hydrogen peroxide (H_2O_2), and MoAb α-M13-HRP conjugate.

Figure 2. Noncompetitive colorimetric calibration ELISA for atrazine using phage particles. ELISA microtiter plates coated with 10 μg mL^{-1} of MoAb K4e7 and a dilution of 1:160 from the original stabilized stock phage suspension (6.25 x 10^9 mL^{-1} phage particles) per well were used. Each point is the average of three replicates.

Figure 3. Adjustment of different parameters for developing PhAIEI assay in optimal conditions. a) Effect of the dilution factor of phage particles on the binding of phage borne clone 13A peptide to MoAb K4e7-MBs complexes in the

presence (●) or absence (■) of 100 ng mL^{-1} of atrazine. The MoAb K4e7 was captured on the MBs at a concentration of 10 μg mL^{-1} and 30 μg, respectively. Each point is the average of two replicated measurements. b) Chronoamperograms of 1 x 10^{-3} M H_2Q and 1 x 10^{-3} M H_2O_2 in CBS solution for three different MoAb K4e7 concentrations: I) 2.5 μg mL^{-1}, II) 5 μg mL^{-1} and III) 10 μg mL^{-1}. The concentrations of atrazine used were: 1) 1 pg mL^{-1}, 2) 100 pg mL^{-1} and 3) 10 x10^3 pg mL^{-1}. A dilution factor of the stabilized stock phage suspension of 1:40 (2.5 x 10^{10} phage particles mL^{-1}) and 30 μg of MBs were used.

Figure 4. Effect of the amount of MBs used on the current obtained. An atrazine concentration of 100 ng mL^{-1} and MoAb K4e7-coated MBs at a concentration of 10 μg mL^{-1} were used. The dilution factor of phage-borne anti-immunocomplex peptide suspension used was 1:40 (which corresponds to 2.5 x 10^{10} phage particles mL^{-1}). Each point is the average of three replicated measurements.

Figure 5. Calibration curve obtained with the PhAIEI assay for atrazine. Curve parameters for the noncompetitive assay were as follows: SC_{50} = 1.3 pg mL^{-1}, LOD = 0.2 pg mL^{-1}, Hill Slope: 0.7 ± 0.1, correlation coefficient for the adjusted equation, R = 0.985. Each point is the average of three replicated measurements. Chronoamperograms recorded for different atrazine concentrations of the calibration curve are shown in the insert of the figure.

Table 1. Accuracy and reproducibility of the atrazine PhAIEI assay

Atrazine concentration (pg mL^{-1})	Intra-assay		Inter-assay	
	Mean	%VC	Mean	%VC
100	96	7.1	91	8.3
1	0.92	6.3	0.91	6.9

Highlights:

We introduce phage particles bearing peptides that recognize an immunocomplex.

We developed a magneto-electrochemical immunosensor for the herbicide atrazine.

Screen printed electrodes are used as electrochemical transducers.

Phage-borne can contribute to the development of ultrasensitive biosensors.

Figure 1

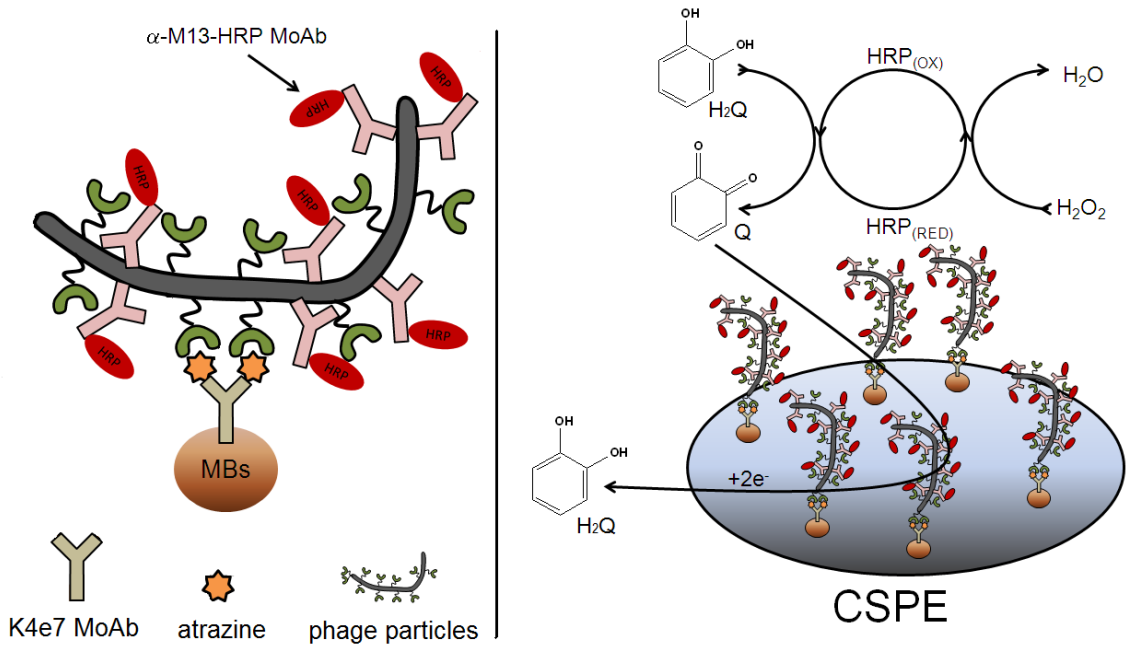


Figure 2

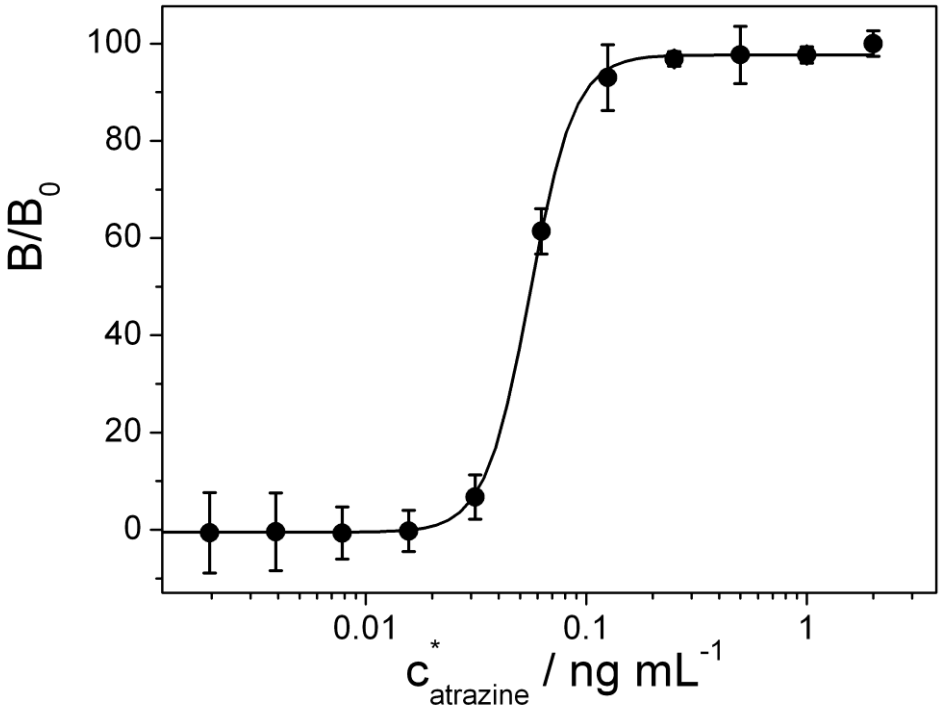
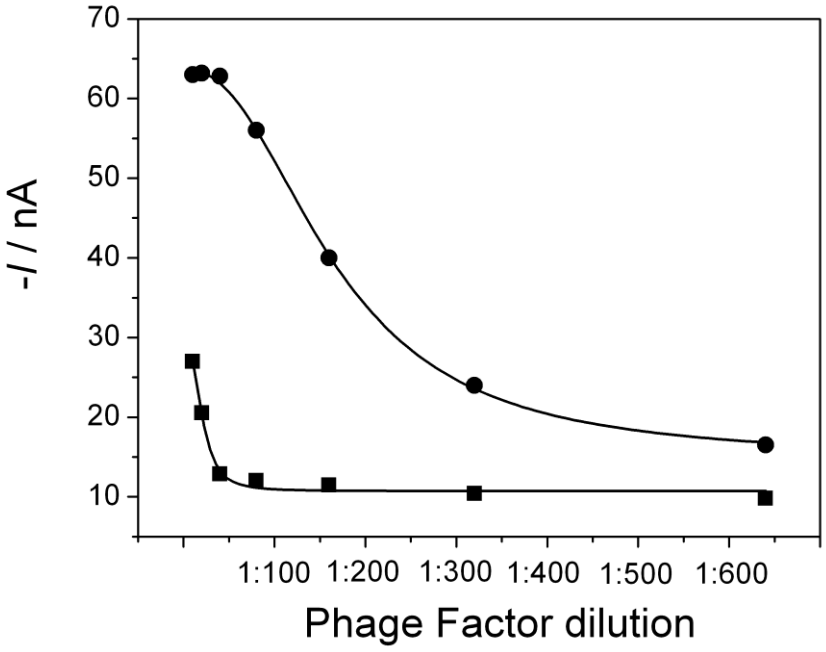


Figure 3

a)



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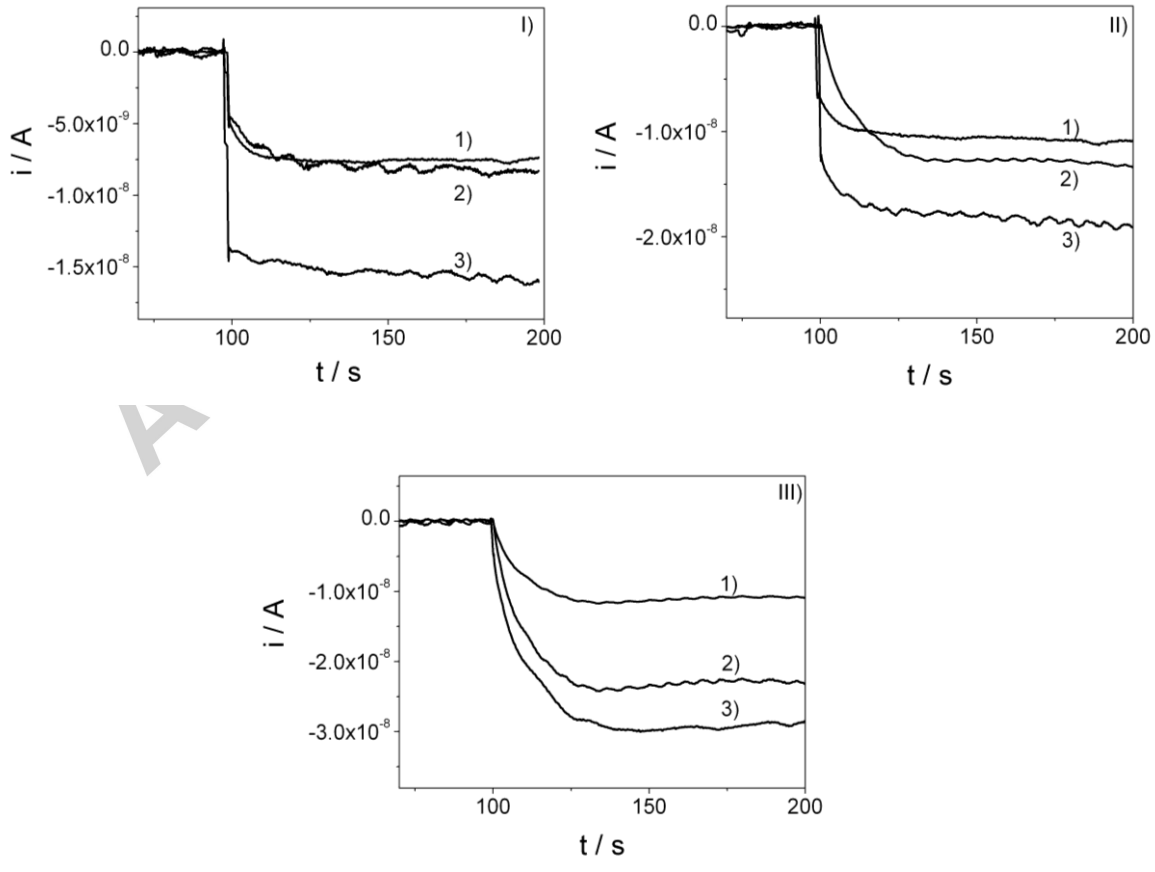

Figure 4

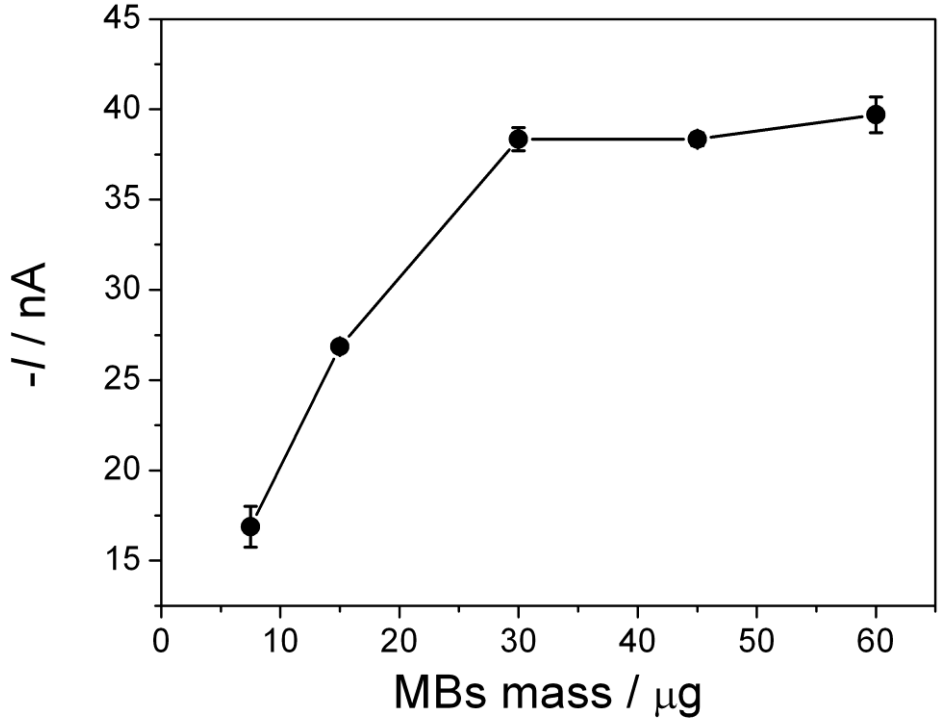

Figure 5