Biosensors and Bioelectronics 32 (2012) 231–237

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Ultra-sensitive electrochemical immunosensor using analyte peptidomimetics selected from phage display peptide libraries

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ARTICLE INFO

Article history:
Received 28 August 2011
Received in revised form 8 December 2011
Accepted 13 December 2011
Available online 21 December 2011

Keywords:
Peptide mimics
Immunosensor
Molinate
Square wave voltammetry

ABSTRACT

Immunosensors for small analytes have been a great addition to the analytical toolbox due to their high sensitivity and extended analytical range. In these systems the analyte is detected when it competes for binding to the detecting antibody with a tracer compound. In this work we introduce the use of phage particles bearing peptides that mimic the target analyte as surrogates for conventional tracers. As a proof of concept, we developed a magneto-electrochemical immunosensor (EI) for the herbicide molinate and compare its performance with conventional formats. Using the same anti-molinate antibody and phage particles bearing a molinate peptidomimetic, the EI performed with an IC_{50} of 0.15 ng mL^{-1} (linear range from 4.4×10^{-3} to 10 ng mL^{-1}). Compared to the conventional ELISA, the EI was faster (minutes), performed with a much wider linear range, and the detection limit that was 2500-fold lower. The EI produced consistent measurements and could be successfully used to assay river water samples with excellent recoveries. By using the same EI with a conventional tracer, we found that an important contribution to the gain in sensitivity is due to the filamentous structure of the phage (9×1000 nm) which works as a multienzymatic tracer, amplifying the competitive reaction. Since phage-borne peptidomimetics can be selected from phage display libraries in a straightforward systematic manner and their production is simple and inexpensive, they can contribute to facilitate the development of ultrasensitive biosensors.

1. Introduction

The enzyme immunoassay with electrochemical detection, which combines the selectivity of the antibody with the sensitivity of electrochemical techniques, has become a powerful tool for the analysis of clinical, environmental, food and commodity samples. Electrochemical techniques are particularly suited for rapid and direct detection of antibody–antigen interactions, which adds to the advantageous high specificity of antibodies that makes possible to eliminate or simplify sample cleanup, making the assay rapid and cost-effective. In immunoassays of small molecules, the analyte competes for binding to a specific antibody with a tracer compound. The tracer typically consists of a structurally related molecule (competing hapten) that provides the binding site, and catalytic molecule that generates the signal (Deshpande, 1996; Gorton, 2005). The synthesis of the tracer is time consuming, and the performance of the assay is greatly influenced by several factors related to the preparation of these conjugates, the final hapten/tracer ratio, the effect of the conjugation chemistry on the tracer enzyme activity, and the need for careful purification of the conjugate from non-conjugated reactants (Cardozo et al., 2005).

An alternative is the use of analyte peptidomimetics expressed on the surface of phage particles. Bacteriophages (phage particles) are viruses that infect bacteria using the host bacteria as a factory for its own replication. A vast repertory of candidate peptides can be expressed in phage-displayed peptide libraries, where randomly generated amino acid sequences are genetically fused to coat proteins 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. We have shown that the antibody used in competitive assays can be readily used as a selector molecule to isolate phage-borne peptidomimetics from a varied panel of phage display peptide libraries. These phage particles have been conveniently used as surrogate tracers for the development of competitive ELISAs for molinate, atrazine, and pyrethroid metabolites (Cardozo et al., 2005; Kim et al., 2008). Once selected, phage can be easily produced in large amounts in an inexpensive way, and perform as a robust reagent

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doi:10.1016/j.bios.2011.12.019

that can withstand harsh conditions (pH 2–12, up to 70 °C and denaturants) without losing activity. In spite of the fact that they do not have enzymatic activity *per se*, they can be detected with the help of commercial anti-phage conjugated antibodies.

On the other hand, the immobilization of antigens or antibodies on a solid surface is a critical step in the construction of the immunosensor, as it plays a fundamental role in what concerns the stability, reproducibility and sensibility of the measured signal (Cosnier, 1999). The detection sensitivity can be increased by controlling the orientation of proteins immobilized on the sensor surface (Liang et al., 2008). Magnetics beads (MBs) with recombinant Protein G covalently bonded to its surface confer a specific binding and orientation of antibodies (Margni, 1996). The use of MBs in separation process offer great advantages such as easy handling, reusability, homogeneous dispersion and a great surface area, which allows great improvement in the separation steps and high reaction kinetics for the antigen-antibody interactions (Aguilar-Arteaga et al., 2010; Font et al., 2008; Lin et al., 2007).

In this work we investigate the use of phage-borne peptides as substitutes for tracers in a heterogeneous competitive immunoassay using a magneto electrochemical immunosensor. The results obtained were compared to those obtained by the conventional ELISA technique. Molinate (*S*-ethylhexahydroazepine-1-carbothioate), a selective pre-emergent herbicide used in rice production, was used as a model analyte. This herbicide is a slightly to moderately toxic compound in EPA toxicity class III, and there is a growing concern about its dissipation from flooded rice fields by drainage of rice paddies or by volatilization into the atmosphere (Cochran et al., 1997; Heath et al., 1997). Using the anti-molinate specific monoclonal antibody (MoAb 14D7) (Rufo et al., 2004) and phage particles expressing a molinate peptidomimetic, an immunosensor was constructed that utilizes protein G functionalized MBs as solid phase for the antibody-molinate-phage reaction, an anti-M13 MoAb coupled to horseradish peroxidase (HRP) for phage detection and pyrocatechol as substrate. The benzoquinone produced by the enzymatic reaction was then detected on a carbon screen printed-electrode (CSPE) by square wave voltammetry (SWV). We found a dramatic increase of sensitivity with regard to the conventional ELISA and a further 6-fold increase when the phage tracer was used as substitute of the conventional hapten-based tracer.

2. Experimental

2.1. Chemicals, antibodies and other reagents

All reagents used were of analytical grade. Molinate was a gift from Stauffer Chemical Co. Development of the monoclonal anti-molinate antibody (MoAb 14D7) has been described by Rufo et al. (2004). S-2-(*p*-aminophenyl)ethyl hexahydroazepine-l-carbothioate conjugated to conalbumin (7bCONA) was a kind gift from Dr. Shirley Gee. Biotinylation of 7bCONA (biot-7bCONA) was performed using the EZ-Link sulfo-NHS-LC-Biotin Kit from Pierce. Streptavidin conjugated to horseradish peroxidase (strep-HRP) was from Pierce. Anti-M13 phage MoAb conjugated to horseradish peroxidase (α-M13-HRP) was purchased from Pharmacia. 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.): 1×10^{-2} M phosphate buffer solutions, 0.137 M NaCl and 2.70×10^{-3} M KCl, pH 7.0 (PBS); 5×10^{-2} M citrate + 5×10^{-2} M phosphate buffer solution, pH 5.00 (CBS), 5×10^{-2} M citrate + 5×10^{-2} M acetate buffer solution, pH 5.5 and PBS containing 0.05% Tween 20 (PBST). H_2O_2 p.a. and H_2SO_4 p.a. were Merck p.a. Water samples were obtained by collecting surface water from the river Rio Cuarto, Rio Cuarto, Argentina, and were spiked with different concentrations of molinate.

2.2. Biopanning of a phage display peptide library with MoAb 14D7

A phage display peptide library previously constructed in our laboratory (Gonzalez-Techera et al., 2008) as fusion with pIII viral coat protein with an estimated diversity of 2×10^8 independent clones was panned with MoAb 14D7. Microtiter plates (Nunc-Immuno Plate Maxi-Sorp) were coated with MoAb 14D7 at 5 μg mL^{-1} in PBS by incubating overnight at 4 °C (100 μL per well). Blocking was performed by completely filling the wells with 1% BSA in PBS and incubating at 37 °C for 1 h. After blocking, each well was washed 5 times with PBS 0.5% Tween-20 (PBST) and the peptide library in PBS 1% BSA was added to 6 precoated wells (approximately a total of 10^{11} phage particles). The plate was incubated for 2 h at 4 °C; wells were washed 10 times with cold PBST to remove unbound phages. Bound phages were eluted by adding 100 μL per well of elution buffer (0.1 N glycine, pH 2.2 adjusted with HCl) and incubating at room temperature for 10 min. Neutralization of pH was done by adding 35 μL of 2 M Tris. The eluted phage (300 μL) were added to 10 mL of log-phase *Escherichia coli* ARI 292 cells and amplified in LB (Luria-Bertani) media containing 0.25% K_2HPO_4, 0.1% $MgSO_4$, 0.1% glucose, and 100 μg mL^{-1} ampicillin to an OD_{600} = 0.4 A.U. M13KO7 helper phage at a multiplicity of infection 10:1 was added. After 30 min at 37 °C, arabinose and kanamycin were added at a final concentration of 0.02% and 40 μg mL^{-1}, respectively, and the cultures incubated overnight at 37 °C with vigorous shaking. This panning protocol was then repeated twice, and after three rounds of panning, individual amplified phage clones were tested for their ability to bind MoAb 14D7 coated wells and to show inhibition of binding to MoAb 14D7 in the presence of molinate. Phage ELISAs were performed as reported before (Cardozo et al., 2005).

2.3. Molinate peptidomimetic bearing phage production and harvest

The phage ELISAs allowed us to choose a phage clone that showed specific inhibition of binding MoAb 14D7 in the presence of 100 ng mL^{-1} of molinate. One bacterial clone was randomly chosen and streaked and grown in a LB agar ampicillin plate. The plasmid DNA carried by this clone was extracted using Qiagen Miniprep Kit and sequenced. The molinate peptidomimetic sequence was CKGLHMWFNC. A single colony was picked up and used for the inoculation with 5 mL of LB media with 100 μg mL^{-1} of ampicillin and grown overnight at 37 °C with vigorous shaking. The next day, a flask with 500 mL of SOP medium (LB media containing 0.25% K_2HPO_4, 0.1% $MgSO_4$) plus 0.1% glucose and 100 μg mL^{-1} of ampicillin, was inoculated with the overnight culture and grown with shaking at 37 °C. After the culture reached an OD_{600} = 0.5 A.U, 100 μL of M13K07 helper phage (New England Biolabs) at a concentration of 1×10^{11} transducing units mL^{-1} was added. The culture was then incubated for 30 min at 37 °C without shaking to allow infection of the cells. Arabinose and kanamycin were then added to a final concentration of 0.02% and 40 μg mL^{-1}, respectively. The cultures were incubated overnight at 37 °C with vigorous shaking. Phage particles from liquid cultures were obtained by clearing the supernatants by centrifugation at 12.000 g for 15 min, precipitated with 0.2 volumes of 20% polyethylene glycol 8000 2.5 M NaCl, (PEG, NaCl), incubated on ice during 1 h, and centrifuged as above. Phage pellets were resuspended in 5 mL of sterile PBS and frozen at −80 °C. Phage particles were titrated by infecting ARI 292 cells with 10-fold serial dilutions of the phage preparation, plating in LB agar

plates containing 100 μg mL^{-1} of ampicillin and counting bacterial colonies.

2.4. Materials and apparatus

The CSPE based on working and counter electrodes of carbon and pseudo-reference electrodes of silver were purchased from Palm Sens. Before use, CSPE surface was electrochemically pretreated (Anjo et al., 1989). Magnetics beads, which facilitate separation and shorten the reaction time, were used as solid surface for EI immunoreactions. The MBs were Dinabeads® (Invitrogen). For convenient orientation of the capture antibody, MBs derivatized with Protein G were used. 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 loaded with saturating amounts of MoAb 14D7 as described below.

Nunc Maxisorp plates (96 well) were purchased from Nunc. The magnetic separator was purchased from Serono Diagnostic.

Cyclic (CV) and square wave (SWV) voltammetric measurements were performed with an AutoLab PGSTAT30 potentiostat. Colorimetric measurements were performed with a Multiskan EX ELISA reader. The samples with MBs were mixed with a Vortemixer Speed Knob vortex.

2.5. Assay procedure for the electrochemical immunosensor

A heterogeneous competitive immunoassay was used for the development of a molinate EI. The schematic representation of the immunoassay is shown in Fig. 1. Molinate and the phage particles compete for a limited amount of MoAb 14D7, which was immobilized on MBs (MoAb 14D7–MBs complexes). Briefly, suspensions of 1.5 μL of MBs were transferred to Eppendorf™ tubes and washed three times with PBS, to remove the NaN_3 preservative. Then, 50 μL of MoAb 14D7 (10 μg/mL in PBS) was added and stirred at 200 rpm at 37 °C for 15 min to obtain MoAb 14D7–MBs complexes. After incubation, a high magnetic field was used for separating the MBs from the supernatant. After discarding the supernatant, the MoAb 14D7–MBs complexes were washed with PBS, eliminating unbound MoAb 14D7. Next, 50 μL of 1% (v/v) mouse serum in PBS was added and incubated for 15 min at 37 °C and stirring at 200 rpm to block free protein G from binding α-M13-HRP MoAb in the last step of the assay. The MBs were separated and washed as described above. The MBs were re-suspended in 50 μL of solution of molinate and phage particles, and stirred to 200 rpm at 37 °C for 15 min (competition step). MBs were then magnetically separated, the supernatant was removed and MBs were washed with PBS. The MBs were resuspended in 50 μL of α-M13-HRP MoAb, at a final dilution of 1:5000 in PBST and stirred at 200 rpm at 37 °C for 15 min. α-M13-HRP MoAb recognizes the presence of phage particles. The MBs were washed with PBS and deposited with the magnet. Next, the MBs were re-suspended in 20 μL of a solution of H_2Q 1×10^{-5} M and H_2O_2 7×10^{-5} M, both in CBS. After 10 min of incubation to 200 rpm at 37 °C, the MBs were magnetically separated and 15 μL of the supernatant was transferred onto the surface of CSPE. It is well known that in the presence of H_2O_2 the enzyme catalyzes the oxidation of H_2Q to Q (Ruan and Li, 2001). Its back electrochemical reduction to H_2Q can be detected on the CSPE through SWV. The peak current ($I_{p,n}$) obtained is proportional to the activity of the enzyme and inversely proportional to the amount of molinate in water river samples. All SWV measurements were performed in the potential range from 0.600 V to 0.000 V, with a square wave amplitude (ΔE_{SW}) of 0.025 V, a staircase step height (ΔE_S) of 0.005 V and a frequency (f) of 25 Hz.

2.6. ELISA protocol for determinations of MoAb 14D7 and phage particles concentrations

Colorimetric checkerboards were performed in order to optimize the MoAb 14D7 and phage particles concentrations on a 96-well high binding microtiter plate, as described by Rufo et al. (2004).Then, 100 μL of MoAb 14D7 from 2-fold serial dilutions starting at 10 μg mL^{-1} and ending in 0.15 μg mL^{-1} were applied to the wells of rows A-H. After 1 h incubation at room temperature, the plates were blocked with 3% (w/v) skimmed milk in PBS for 30 min and washed with PBST. Then, 100 μL of 2-fold serial dilutions of phage starting at 2×10^{10} particles mL^{-1} were dispensed to columns 1–11 of the microtiter plate. These were incubated for 1 h at room temperature with gentle rocking. After a washing step with PBST, 100 μL of a 1:5000 dilution of α-M13-HRP MoAb in PBST was added to each well. After 1 h, the plates were washed with PBS and 100 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 15–20 min by the addition of 50 μL of 2 M H_2SO_4, and the absorbance at 450 nm (corrected at 600 nm) was read.

2.7. ELISA protocol for determinations of competitor hapten (biot-7bCONA) for molinate conventional determination

Optimization of MoAb 14D7 and biot-7bCONA concentrations were performed on a 96-well high binding microtiter plate. ELISA plates were coated with 100 μL of MoAb 14D7. Two-fold serial dilutions starting at 10 μg mL^{-1} and ending in 0.15 μg mL^{-1} were applied to the wells of rows A–H. After incubating for 1 h at 37 °C the wells were washed with PBS and blocked with 1% (w/v) BSA in PBS. Later, the wells were washed with PBST. Two-fold serial dilutions of biot-7bCONA starting at 8 μg mL^{-1} were added to columns 1–11 and incubated for 1 h at room temperature. Wells were washed with PBST and 100 μL of strep-HRP (1:5000 dilution in PBS) were added and incubated for 1 h at room temperature. After washing with PBST, HRP substrate was dispensed into each well and the absorbance was read as described above.

3. Results and discussion

3.1. Microtiter plate ELISA for molinate using phage-borne peptidomimetics

As a reference for comparison, we initially optimized the performance of an ELISA assay for molinate using a selected phage particle. The antibody coating concentration and phage dilution were selected by checkerboard titration and the α-M13-HRP was used for detection. The titration curve is shown as binding percentage (B/B_0 = absorbance value/absorbance value with no inhibition) *vs* molinate concentration (c^*_{Mo}) using a logarithmic scale for c^*_{Mo} (Fig. 2), and was fitted to a four-parameter logistic equation according to the following formula (Rodbard, 1974):

$$y = D + \frac{A - D}{[1 + 10\exp((\log IC_{50} - \log c^*_{Mo})(\text{Hill Slope}))]} \quad (1)$$

where A and D are the maximum and minimum B/B_0 values, respectively, IC_{50} is the concentration of molinate which produces 50% inhibition, c^*_{Mo} is the molinate concentration and Hill Slope is the slope at the midpoint of the sigmoid curve. The limit of detection (LOD), calculated as the concentration of molinate causing a drop of biding percentage equal to three times the standard deviation of the blank (ACS Committee on Environmental Improvement, 1980), was 11.0 ng mL^{-1}. The highest sensitivity, IC_{50} = 43 ng mL^{-1}, was

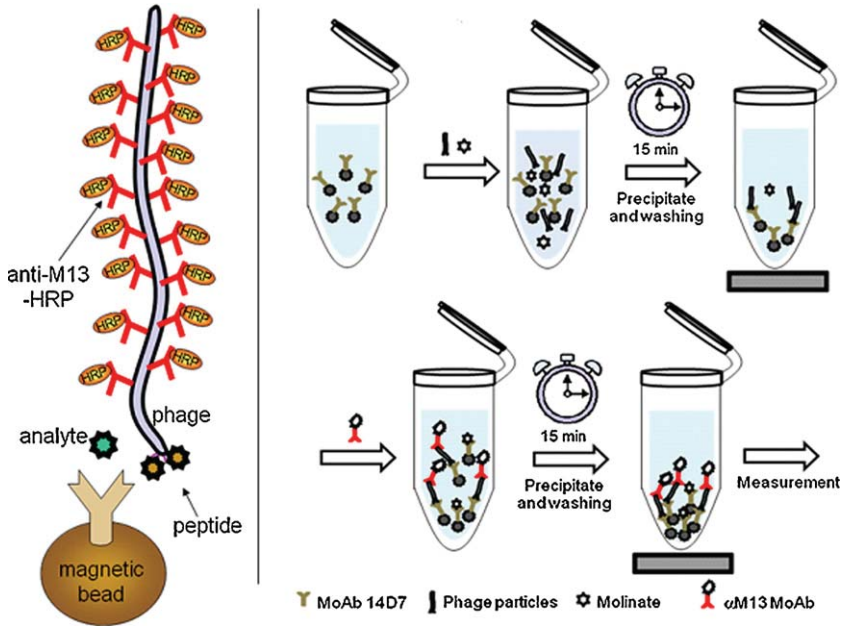

Fig. 1. Schematic representation of molinate immunosensor. Left panel: immunoassay components. Right panel: immunoassay steps previous to the electrochemical measurement.

attained using 1 μg mL^{-1} of MoAb 14D7 for coating, and 1.2×10^8 phage particles per well. The same values of antibody concentration and phage dilution were adopted for the EI development.

3.2. Electrochemical magneto immunosensor

Different parameters were studied to optimize the performance of the EI, including: the activation of CSPE, the concentration of MoAb 14D7 and phage particles, the amount of MBs, volume and concentration of redox mediator and enzymatic substrate solutions.

3.2.1. CSPE optimization

We used an CSPE to study the cyclic voltammogram corresponding to the oxidation of H_2Q to Q and the reduction of Q back to H_2Q, a quasi-reversible two-electron redox process, (Forzani et al., 1997). Initially, we used an untreated electrode, which showed a poor performance when tested to monitor the oxidation/reduction of 1×10^{-3} M of H_2Q in CBS (results not shown). Then, a pre-treatment was applied to the working electrode, consisting of an electro-oxidation of the CSPE surface in 0.01 M KOH (Anjo et al., 1989). After this treatment (Section 2.4), the cyclic voltammogram obtained for the activated CSPE showed a well-defined anodic peak ($E_{p,a}$ = 0.364 V) and its corresponding cathodic peak ($E_{p,c}$ = 0.110 V) when the scan rate was reversed. On the other hand, after activation, a reproducibility test was performed measuring the reduction of Q electrochemically generated, using a 1×10^{-5} M $H_2Q + 7 \times 10^{-5}$ M H_2O_2 in CBS and the parameters mentioned above (Section 2.5). The variation coefficient (CV) was determined from measurements of net peak currents ($I_{p,n}$) obtained for each CSPE, giving a value of CV = 7.50%, which indicates the good reproducibility of CSPE.

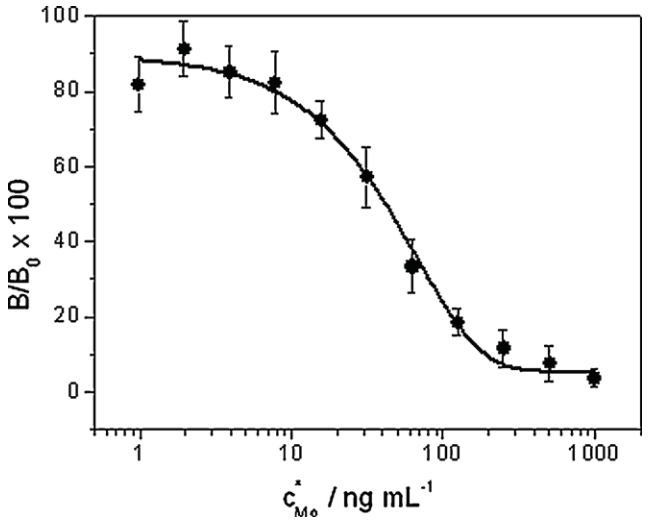

Fig. 2. ELISA competitive assay for molinate using phage particles. $c^*_{14D7MoAb}$ = 1 μg mL^{-1}; 1.2×10^8 phage particles per well. Each point is an average of four replicates.

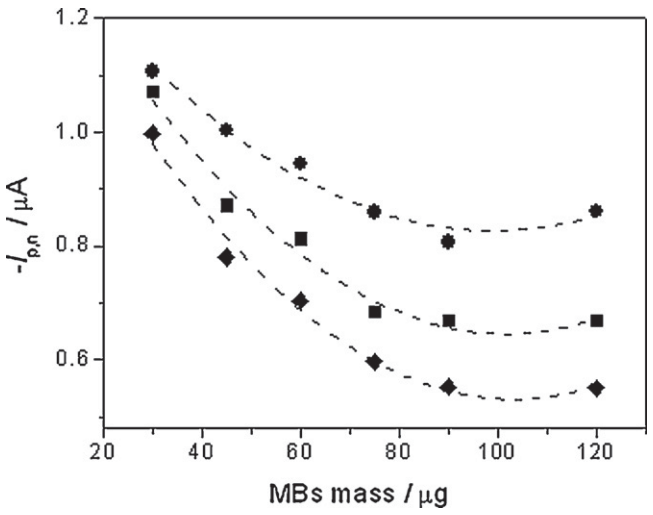

Fig. 3. Effect of the amount of MBs and antibody concentration on $I_{p,n}$. $c^*_{14D7MoAb}$: (♦) 1 μg mL^{-1}, (■) 2.5 μg mL^{-1} and (●) 5 μg mL^{-1}. Each point is the average of two replicated measurements.

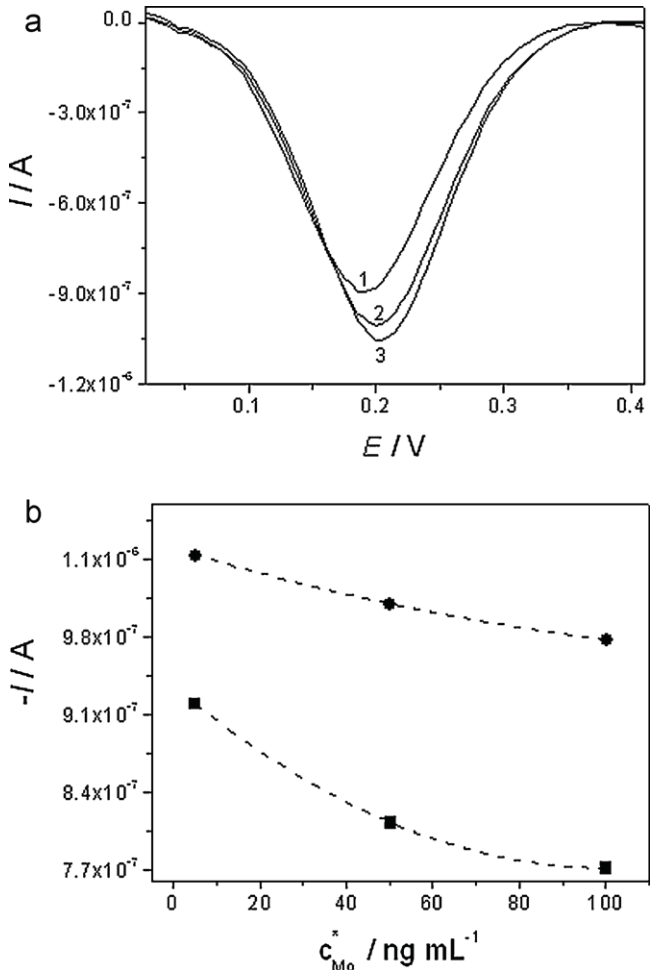

Fig. 4. (a) SWV of 1×10^{-5} M H_2Q and 7×10^{-5} M H_2O_2 in CBS solution for three different phage particles concentrations: (1) 0, (2) 1.2×10^9 and (3) 2.4×10^9 phage particles mL^{-1}. (b) $I_{p,n}$ *vs.* c^*_{Mo} for two phage concentrations: (■) 1.2×10^9 and (●) 2.4×10^9 phage particles mL^{-1}. Each point is an average of three replicated measurements.

3.2.2. Optimization of the magnetic beads-MoAb 14D7 ratio to be used in the EI

This study was performed by varying the MBs amount from 30 to 120 μg (0.5 to 40 μL of MBs solution) in combination with different concentrations of MoAb 14D7 and 1.2×10^8 phage particles mL^{-1} (Fig. 3). The increase in the number of MBs produced a decrease of the $I_{p,n}$ reaching steady-state current at 75 μg of MBs. All curves have a similar behavior, but the $I_{p,n}$ was slightly higher when the antibody concentration was 5.0 μg mL^{-1}. In order to have a better control on the current and promote a more efficient interaction between all components, 45 μg (1.5 μL) of MBs and a concentration of 5 μg mL^{-1} of MoAb 14D7 were chosen for all experiments. The effect of the incubation time was also studied. We observed that the $I_{p,n}$ value increased up to 15 min, then it remained basically constant (data not shown). This time was therefore adopted for all other experiments.

3.2.3. Optimization of phage particles concentration used for the electrochemical magneto immunosensor

Based on the results of the ELISA optimization, two phage concentrations were tested: 2.4×10^9 and 1.2×10^9 particles mL^{-1}. The square wave voltammograms obtained are shown in Fig. 4a. The largest phage concentration produced the greater $I_{p,n}$ values. However, when we studied the variation of the $I_{p,n}$ as a function of the analyte concentration, we found that the more diluted phage produced the largest difference in $I_{p,n}$ between molinate concentrations (Fig. 4b). This phage concentration while producing a lower $I_{p,n}$ value allowed to achieve a more sensitive response to variations in the analyte concentration, and was used for all experiments.

Table 1
Acurracy and precision of the molinate EI.

Molinate concentration (ng mL^{-1})	Intra-assay		Inter-assay	
	Mean	%VC	Mean	%VC
0.1	0.107	4.1	0.112	6.1
1	1.05	4.8	1.10	5.9

3.2.4. Optimization of the enzymatic substrate and redox mediator volumes and concentrations

In order to optimize the $I_{p,n}$ values, H_2Q and H_2O_2 concentrations were evaluated. A small H_2Q concentration permitted to use a small H_2O_2 concentration and avoid the HRP inactivation (Arnao et al., 1990). Then, it was necessary to reduce the concentration of H_2Q without affecting the sensitivity of the technique. After exploring different concentrations we found that 1×10^{-5} M H_2Q in CBS worked as a convenient trade-off. In order to ensure that the enzyme reaction rate depends only on the H_2O_2 concentration, the H_2O_2 concentrations were varied from 5×10^{-6} to 1×10^{-4} M. $I_{p,n}$ values increase and a plateau was reached for 7×10^{-5} M H_2O_2 concentration (data not shown). This H_2O_2 concentration was then used for all experiments. Additionally, the reaction volume added to CSPE was also studied. The $I_{p,n}$ increases with volume reduction, due to an increment of concentration of enzymatically produced Q. In this way a 20 μL reaction volume was chosen. Since the minimum volume required to cover CSPE is 15 μL, volumes below 20 μL were not used.

3.2.5. Analytical performance of the EI for molinate

Using the optimized parameters, a dose-response titration for molinate was carried out in the 1×10^{-4} to 1×10^3 ng mL^{-1} range (Fig. 5a). The calibration curve was plotted as binding percentage (B/B_0) *vs* c^*_{Mo}, using a logarithmic scale for c^*_{Mo}, were B_0 is the maximum $I_{p,n}$ obtained without analyte, and B is the $I_{p,n}$ obtained for the competitive reaction. The calibration curve was fitted using Eq. (1), exhibiting a linear range from 0.73×10^{-2} to 10 ng mL^{-1}. The IC_{50} value was 0.150 ng mL^{-1}, the LOD value 0.0044 ng mL^{-1}, with a Hill Slope of -0.458 ± 0.034. This Hill Slope value agrees with the linear range of concentrations (three orders of magnitude) that can be determined. The LOD was about 2500-fold better than that of conventional ELISA (Section 3.1). The IC_{50} value was about 290-fold better than that obtained for the ELISA format (Section 3.1) and 450-fold better than the IC_{50} of 69 ng mL^{-1} obtained previously using the same antibody and a conventional chemical hapten (Rufo et al., 2004).

The accuracy and precision of the EI was checked using standard solutions of the herbicide at 0.1 and 1.0 ng mL^{-1}. The intra-assay parameters were tested by performing five consecutive measurements of the same sample. These measurement series were repeated for 3 consecutive days to estimate the inter-assay values. The results obtained are summarized in Table 1. The molinate assay showed an excellent accuracy and precision.

3.3. Comparison of the molinate EI sensor using phage or conventional hapten tracers

The performance of the EI was compared to that of the EI set up with a conventional tracer (biot-7bCONA) using strep-HRP conjugate for detection. By ELISA checkerboard titration (Section 2.7) the appropriate concentrations of MoAb 14D7 as coating (1.25 μg mL^{-1}) and biot-7bCONA (0.25 μg mL^{-1} as competing

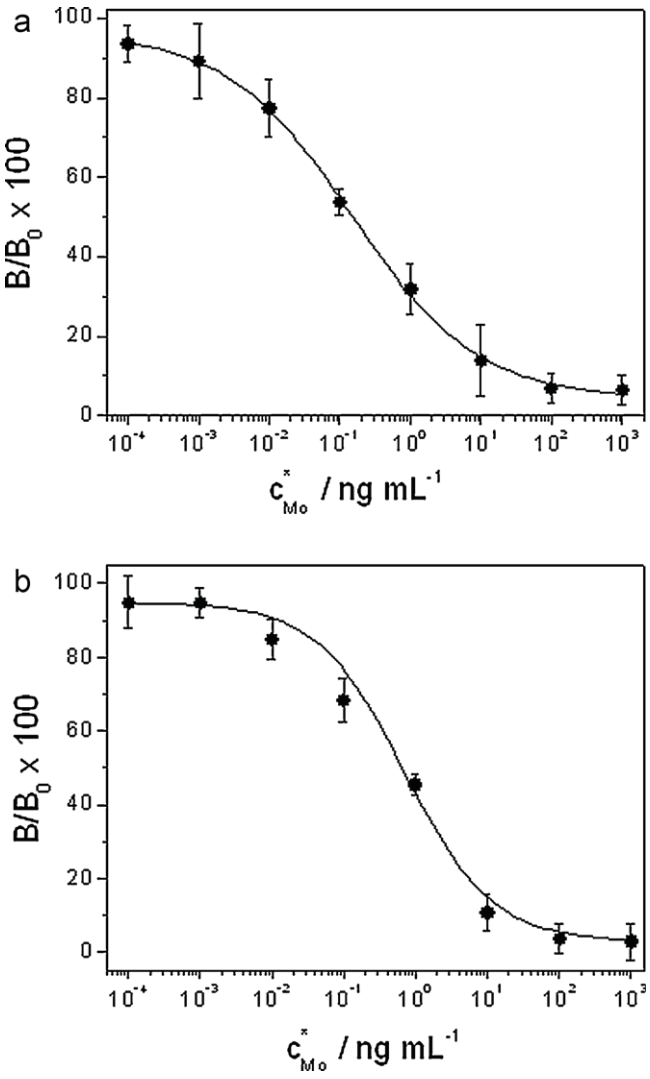

Fig. 5. EI calibration curve for molinate obtained using phage particles or the conventional chemical hapten. Each point is the average of three replicated measurements. (a) Curve parameters for the phage assay: $IC_{50} = 0.150\ ng\ mL^{-1}$, Hill Slope: -0.458 ± 0.034, $R = 0.956$. (b) Curve parameters for the conventional assay set up with biot-7bCONA: $IC_{50} = 0.916\ ng\ mL^{-1}$, Hill Slope: -0.401 ± 0.05, $R = 0.961$.

tracer) were selected. The competitive ELISA assay set up using these conditions (data not shown) performed with an IC_{50} value of $44.0\ ng\ mL^{-1}$, which is highly similar to that obtained using phage particles (Section 3.1). This antibody–tracer system was then adapted into the EI format, using the antibody and biot-7bCONA concentrations optimized by ELISA. The dose-response curve is displayed in Fig. 5b, showing a linear range from 0.1 to $10\ ng\ mL^{-1}$. The IC_{50} value was $0.916\ ng\ mL^{-1}$, and the Hill Slope was -0.410 ± 0.05. The LOD was $0.041\ ng\ mL^{-1}$, which is about one order of magnitude bigger than that obtained with the phage EI (LOD $0.0044\ ng\ mL^{-1}$). Since the same antibody was used in both EI, we may explain this finding by the filamentous nature of the phage that allows accommodating a large number of α-M13-HRP conjugate molecules (Fig. 1); therefore, for each analyte molecule that inhibits the binding of the peptidomimetic, there is a big loss of enzymatic activity generating a significant signal drop. In addition, this high signal-to-binding ratio allows to use small amounts of phage as reference ligand (tracer) without losing the signal, which in turn, is known to result in lower IC50 values.

3.4. Molinate measurement in river water samples using the phage EI

The usefulness of the immunosensor for the analysis of real samples was demonstrated by analyzing river water samples obtained from the Río Cuarto river. Non-spiked river samples showed zero readings when analyzed by ELISA or EI. Then molinate was spiked at 0.1 and $10\ ng\ mL^{-1}$. Each sample was diluted ten times with PBS and analyzed in triplicates. Values of 0.011 ± 0.002 and $1.02 \pm 0.06\ ng\ mL^{-1}$ were obtained for both spiked samples, respectively, which correspond to recoveries of $110 \pm 5\%$ and $102 \pm 6\%$, respectively. These results suggest that our EI can be used to determine very low concentrations of molinate in river water with high recoveries and without the need of sample treatment.

4. Conclusions

In this work we developed a magneto-electrochemical immunosensor that uses an innovative element in immunoelectrochemical detection of trace amount of small compounds, namely viral particles expressing analyte peptidomimetics. As observed before with other systems, the transition from the conventional microtiter ELISA, to the electrochemical format was accompanied by a major improved in sensitivity and an extended dose-response range. Using the same antibody, the EI showed 290-fold IC_{50} reduction and the linear range expanded from 1 to 3 orders of magnitude, which brought the LOD to $0.0044\ ng\ mL^{-1}$, about 2500-fold lower than that of the ELISA assay. Interestingly, by comparison with the conventional EI format, we could also demonstrate that the filamentous nature of the viral particle is an important contribution to the sensitivity of the phage EI. In this way, phage expressing analyte peptidomimetics behave as convenient nanoparticles for immunosensor development, because: (a) they can be selected straightforward by well-established methods of phage display, (b) once isolated, they can be produced in large amounts and inexpensively in *E. coli* cultures, (c) the phage particles are used directly avoiding the preparation of chemical conjugates, (d) they provide a large surface that can be chemically modified with fluorophores, biotin, enzymes, *etc.* to generate tracers with the desire properties an a high signal to binding ratio. The use of phage particles in immune electrochemical assays appears as a versatile and promising tool for further developments in the field.

Acknowledgments

Financial supports from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaría de Ciencia y Técnica (SECyT) from the Universidad Nacional de Río Cuarto, NIH Fogarty International Center Grant TW05718, FMV 3138 from ANII, Uruguay, CYTED 109AC0371, are gratefully acknowledge. F.J. Arévalo acknowledges to CONICET for post-doctoral research fellowships.

References

ACS Committee on Environmental Improvement, 1980. Anal. Chem. 52, 2242–2249.
Aguilar-Arteaga, K., Rodriguez, J.A., Barrado, E., 2010. Anal. Chim. Acta 674, 157–165.
Anjo, D.M., Kahr, M., Khodabakhsh, M.M., Nowinski, S., Wanger, M., 1989. Anal. Chem. 61, 2603–2608.
Arnao, M.B., Acosta, M., del Rio, J.A., Varón, R., García-Cánovas, F., 1990. Biochim. Biophys. Acta 1041, 43–47.
Cardozo, S., González-Techera, A., Last, J.A., Hammock, B., Kramer, K., González-Sapienza, G.G., 2005. Environ. Sci. Technol. 39, 4234–4241.
Cochran, R.C., Formoli, T.A., Pfeifer, K.F., Aldous, C.N., 1997. Regul. Toxicol. Pharmacol. 25, 146–157.
Cosnier, S., 1999. Biosens. Bioelectron. 14, 443–456.
Deshpande, S.S., 1996. Enzyme Immunoassays, from Concept to Product Development. Chapman & Hall, New York.

Font, H., Adrian, J., Galve, R., Estévez, M.C., Castellari, M., Gratacós-Cubarsí, M., Sánchez-Baeza, F., Marco, M.P., 2008. J. Agric. Food Chem. 56, 736–743.
Forzani, E.S., Rivas, G.A., Solís, V.M., 1997. J. Electroanal. Chem. 435, 77–84.
Gonzalez-Techera, A., Umpiérrez-Failache, M., Cardozo, S., Obal, G., Pritsch, O., Last, J.A., Gee, S.J., González-Sapienza, G., 2008. Bioconjug. Chem. 19, 993–1000.
Gorton, L. (Ed.), 2005. Biosensors and Modern Biospecific Analytical Techniques. , 44th ed. Elsevier, Amsterdam, Netherlands.
Heath, A.G., Cech Jr., J.J., Brink, L., Moberg, P., Zinkl, J.G., 1997. Ecotoxicol. Environ. Saf. 37, 280–288.
Kim, H.J., González-Techera, A., González-Sapienza, G.G., Ahn, K.C., Gee, S.J., Hammock, B.D., 2008. Environ. Sci. Technol. 42, 2047–2053.
Liang, K-Z., Qi, J-S., Mu, W-J., Chen, Z-G., 2008. J. Biochem. Biophys. Methods 70, 1156–1162.
Lin, Y.Y., Liu, G., Wai, C.M., Lin, Y., 2007. Electrochem. Commun. 9, 1547–1552.
Margni, R.A., 1996. Inmunología e Inmunoquímica. Fundamentos 5ta. Panamericana, Buenos Aires.
Rodbard, R., 1974. Clin. Chem. 10, 1255–1270.
Ruan, C., Li, Y., 2001. Talanta 54, 1095–1103.
Rufo, C., Hammock, B.D., Gee, S.J., Last, J.A., González-Sapienza, G.G., 2004. J. Agric. Food Chem. 52, 182–187.
Scott, J.K., Smith, G.P., 1990. Science 249, 386–390.