Biosensors and Bioelectronics 64 (2015) 650–656

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Development of a highly sensitive noncompetitive electrochemical immunosensor for the detection of atrazine by phage anti-immunocomplex assay

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ARTICLE INFO

Article history:
Received 4 July 2014
Received in revised form
17 September 2014
Accepted 20 September 2014
Available online 28 September 2014

Keywords:
Anti-immunocomplex peptide
Immunosensor
Atrazine
Chronoamperometry

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.

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

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http://dx.doi.org/10.1016/j.bios.2014.09.046
0956-5663/

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 $1 \times 10^{9-10}$ 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, 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.): 1×10^{-2} M $KH_2PO_4 + Na_2HPO_4$, 0.137 M NaCl and 2.70×10^{-3} M KCl, pH 7.0 (phosphate buffer solution, PBS); 5×10^{-2} M $Na_3C_3H_5O(COO)_3 + 5 \times 10^{-2}$ M Na_2HPO_4, pH 5.00 (citrate buffer solution, CBS); 5×10^{-2} M $Na_3C_4H_5O_4 + 5 \times 10^{-2}$ 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 purchased from 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 Ecochemie, 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