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An electrochemical competitive biosensor for ochratoxin A based on a DNA biotinylated aptamer

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ABSTRACT

Ochratoxin A (OTA) is one of the most important mycotoxin contaminants of foods, particularly cereals and cereal products, with strict low regulatory levels (of ppb) in many countries worldwide. An electrochemical competitive aptamer-based biosensor for OTA is described. Paramagnetic microparticle beads (MBs) were functionalized with an aptamer specific to OTA, and were allowed to compete with a solution of the mycotoxin conjugated to the enzyme horseradish peroxidase (OTA–HRP) and free OTA. After separation and washing steps helped with magnetic separations, the modified MBs were localized on disposable screen-printed carbon electrodes (SPCEs) under a magnetic field, and the product of the enzymatic reaction with the substrate was detected with differential-pulse voltammetry. In addition to magnetic separation assays, other competitive schemes (direct/indirect aptasensors performed on the SPCEs surface or using gold nanoparticles functionalized with the aptamer) were preliminary tested, optimized and compared. The magnetic aptasensor showed a linear response to OTA in the range 0.78–8.74 ng mL^{-1} and a limit of detection of 0.07 ± 0.01 ng mL^{-1}, and was accurately applied to extracts of certified and spiked wheat samples with an RSD lower than about 8%.

1. Introduction

Ochratoxin A (OTA) is frequently present in various agricultural commodities during storage (Bayman and Baker, 2006). At least nine ochratoxins have been identified, mainly comprising the A, B and C ochratoxins, but OTA is the most prevalent, toxic, and relevant toxin of the group (Rai and Varma, 2010).

The highest levels of OTA are usually found in cereal grains (corn, barley, wheat and rye). However, the contamination of OTA in derived cereal products and other foods such as beans and soluble coffee, dried fruits, grape juice and must, nuts, baby foods, and wine has become a matter of great concern, as it is responsible for chronic diseases in humans and animals (Rai and Varma, 2010). The highest OTA concentration in unprocessed cereals and cereal products permissible by the European Community is 3–5 μg kg^{-1} (Commission Regulation No. 1881/2006).

The determination of OTA is essential to minimize the consumption of contaminated foods. Many of the analytical methods for OTA in foodstuffs have been validated in collaborative studies of the AOAC (Monaci and Palmisano, 2004; Cigic and Prosen, 2009). These usually use liquid extraction, solid-phase extraction or immunoaffinity columns for the extraction and cleanup of the sample, and high-performance liquid chromatography with fluorescence detection (HPLC–FLD) for determination (Monaci and Palmisano, 2004; Cigic and Prosen, 2009), obtaining limits of detection below 0.1 μg kg^{-1}. However, HPLC–FLD methods require sophisticated instrumentation and expertise.

Simple immunoassay methods, such as the enzyme-linked immunosorbent assays (ELISA), are also very popular (Visconti and De Girolamo, 2005). Some of them reach the sensitivity required for specific applications, although most are commonly used as a screening technique. The main disadvantages of the ELISAs are the time necessary for the assays, the high volumes in comparison with e.g. biosensor assays and the frequent negative or positive false screening results. Immunoaffinity columns and ELISA kits devoted to OTA analysis are also commercially available (European Mycotoxin Awareness Network, 2010). More recently, the first biosensors for OTA have appeared, mainly involving fluorescence (Prieto-Simon et al., 2007), electrochemical (amperometric and impedimetric) (Bonel et al., 2010; Alarcon et al., 2004; Radia et al., 2009), piezoelectric QCM (Vidal et al., 2009), or SPR (surface plasmon resonance) immunosensors (Shankaran et al., 2007). From all these kind of biosensors for OTA, electrochemical inmunosensors have the advantages of simplicity and sensitivity.

Selected aptamers for OTA were recently described for the first time, following characterization by fluorescence polarization and equilibrium dialysis (Cruz-Aguado and Penner, 2008a,b), and were demonstrated to be useful for the determination of OTA in wheat

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grains by affinity chromatography coupled to molecular fluorescence detection (Cruz-Aguado and Penner, 2008b). The binding affinities of the selected OTA aptamers, determined by equilibrium dialysis, are in the nanomolar range (Cruz-Aguado and Penner, 2008b), comparable to or below the binding constants of antibodies to OTA, and they are very selective to OTA target molecule, for which they can be used as useful biorecognition elements in biosensors for the mycotoxin. Since 1995, various aptamers have been applied to electrochemical biosensors with large molecular targets (proteins), but few with small targets (i.e. cocaine, adenosine or theophilline) (Alan et al., 2009; Tombelli and Mascini, 2009). The interest in aptamers (sometimes described as chemical antibodies) originates from their well known advantages over antibodies: specificity, smaller size (molecular mass 5–15 kDa), easier in vitro production (including toxic compounds), chemical, thermal and biological strength (Willner and Zayats, 2007; Velasco-Garcia and Missailidis, 2009; Stoltenburg et al., 2007), and a highly reproducible binding ligand structure.

Sandwich-type assays are one of the commonly used protocols for electrochemical aptasensors of large molecules (Alan et al., 2009; Tombelli and Mascini, 2009; Willner and Zayats, 2007; Velasco-Garcia and Missailidis, 2009; Sadik et al., 2009; Sassolas et al., 2009; Centi et al., 2008, 2009a,b; Tombelli et al., 2007), although this strategy is not suitable for small sized molecules (e.g. for OTA). Recently, label-free electrochemical aptasensors have emerged through the use of faradaic impedance spectroscopy (EIS), following changes in electron-transfer resistance at the electrode resulting from complexation of the aptamer (Shankaran et al., 2007; Deng et al., 2009). However, the use of EIS to identify affinity complexes between aptamers and small molecules (e.g. OTA) is difficult, since the reorganization of the aptamer–target complex on the electrode yields a minute change in the interfacial electron-transfer resistance compared to the free aptamer-modified electrode.

In a very recent report, an electrochemical aptamer-based sensor is described, where three single-stranded DNA molecules were immobilized on a glassy-carbon electrode for measuring the redox current of a methylene blue electrochemical probe in a manner that was dependent on OTA concentration (Kuang et al., 2010). Nevertheless, preparation of the modified non-disposable electrode (only one measurement) is hard to complete, and the complex sensing process depends on an induced conformational change of the aptamer, repetitive non-specific to OTA hybridization steps, and low-sensitive cyclic voltammetric transduction.

In previous studies in our laboratory, we have developed piezoelectric (Vidal et al., 2009) and amperometric immunosensors with polyclonal (Bonel et al., 2010) and monoclonal (Vidal et al., 2011) antibodies against OTA, providing the antibodies high selectivity to OTA from the wheat matrix and the voltammetric transduction high sensitivity (LOD 0.20 ng mL^{-1} OTA) to biosensors. We have also reported a sensitive competitive electrochemical aptasensor to C-reactive protein (Centi et al., 2009a). In this work, we examined the capabilities of competitive assay schemes on paramagnetic microbeads applied to electrochemical aptasensors for the mycotoxin OTA. Traditionally, magnetic separation has been used in electrochemical DNA biosensors based on hybridization, as a good way to collect another nucleic acid target, i.e. DNA or RNA (Willner and Zayats, 2007; Velasco-Garcia and Missailidis, 2009), but they have rarely been used in aptasensor assays (Centi et al., 2008, 2009a,b; Tombelli et al., 2007). Using a magnetic field, the beads are easily isolated, allowing very efficient capture, washing and detection steps. An external magnet was then used to concentrate the modified MBs close to the electrode surface and to separate them from other reagents involved in the analytical processes for OTA, avoiding unspecific adsorptions. The aptamer-based biosensor is very selective and has slightly higher sensitivity than a monoclonal-based biosensor. The aptasensor was validated using certified wheat samples.

2. Materials and methods

2.1. Apparatus

Voltammetric measurements were carried out with a computer controlled AutoLab PGSTAT-12 potentiostat (Utrecht, The Netherlands) connected to three electrode screen-printed carbon electrodes (SPCEs). The area of the working electrode in SPCEs was 0.13 cm^2, and they were printed from a carbon-based ink (Gwent, C2091208D1). A pseudo reference electrode was made printing a silver-based ink, and the auxiliary electrode was from a carbon ink. Magnetic separation stands (ref. Z5342, twelve-position, 1.5 mL volume) were from Promega (Madison, WI, USA). The intra-batch variation of sensitivity among ten lots of SPCEs, measured by chronoamperometry of 1 mg L^{-1} p-benzoquinone (p-BQ), was always less than about 5% (%CV, $n = 10$).

2.2. Chemicals

The 36-mer oligonucleotide sequence used as the aptamer probe for OTA, functionalized in position 5′ with biotin (5′bi-AptOTA), was supplied by Aptares AG (Mittenwalde, Germany). Ochratoxin A (99+%) was supplied by Acros Organics. Ochratoxin B (OTB) was obtained from Sta. Cruz Biotechnology. Streptavidin magnesphere© paramagnetic beads (saMBs, containing approximately 1 mg mL^{-1} magnetic particles in PBS, diameter 1.0 ± 0.5 μm) were purchased from Promega. Horseradish peroxidase (HRP), bovine serum albumin (BSA, 98%), extravidin–peroxidase conjugate (saHRP, concentration 2.1 mg mL^{-1}, molar ratio 0.8), streptavidin–gold nanoparticles from Streptomyces avidinii (saAuNPs, S9059, suspension in 0.01 M PBS buffer, diameter 10 nm), biotin (99%), casein from bovine milk, warfarin (>98%), L-phenylalanine (L-Phen), 1-hydroxy-2-naphthoic acid (HNA) and hydroquinone were purchased from Sigma–Aldrich (Madrid, Spain). The composition of the buffers used were as follows:

Buffer A, used for washing beads: SSC 0.5× (0.075 M NaCl and 0.0075 M trisodium citrate dihydrate), pH 7.2.
Buffer B, used for binding the aptamer to saMBs: 5 mM Tris–HCl, 0.5 mM EDTA, 1 M NaCl, pH 7.5.
Buffer C, for competition step: 10 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 20 mM $CaCl_2$, pH 8.5.
Buffer D, used for electrochemical detection: 10 mM HEPES, 2 mM $CaCl_2$, pH 7.4.
PBS buffer: 0.1 M phosphate and 0.1 M KCl, pH = 7.4.
PBST buffer: PBS containing 0.05% (w/v) of Tween-20.

The OTA certified reference wheat materials used are detailed in supporting info (section SI-1).

Caution: OTA is a potent carcinogen and extreme caution is therefore necessary to avoid contact with it. Contaminated materials of OTA, OTB, AuNPs and warfarin must be appropriately disposed.

2.3. Immobilization of the aptamer on the magnetic beads

The immobilization of the 5′bi-AptOTA on the streptavidin-coated paramagnetic beads (saMBs) was based on the very strong streptavidin–biotin interaction ($K_d \sim 10^{-15}$). The modified beads (AptOTA-MBs) were reacted with a biotin solution to block all remaining active sites, and then washed and resuspended in 50 μL of buffer C (for details, see supporting info, section SI-1).

2.4. Competitive step of the aptasensor using MBs

A 50-μL sample of the AptOTA-MBs was mixed in an eppendorf vial with 200 μL of a previously mixed solution containing OTA (variable concentrations in the range 0–1000 ng mL^{-1}) and the conjugate OTA–HRP (a fixed concentration of 1 μg mL^{-1}). The mixed anhydride method was used for preparing the OTA–HRP conjugate (see details in the supporting info appendix, SI-1 section). After 15 min of incubating at room temperature, the vial was positioned on a magnetic block to allow the precipitation and separation of the modified MBs at the bottom, the supernatant was removed, and the beads were washed twice using buffer D (containing 0.1%, w/v, of Tween-20).

2.5. Electrochemical detection

A 10-μL sample of the beads was dropped onto the surface of the SPCE. For the specific location of the beads close to/on the electrode surface, a magnetic block (Idemag, Spain, model NS-35, 12,200 G) was placed just at the bottom of the SPCEs. Then 5 μL of 18 mM hydroquinone and 45 μL of 1.1 mM hydrogen peroxide in HEPES buffer was deposited (in this order) onto the SPCE strip, and after 10 min at room temperature the enzymatic product (p-BQ) was determined using highly sensitive differential-pulse voltammetry (DPV, potential ranging from +200 to −400 mV, step potential 2 mV, scan rate 10 mV s^{-1}, modulation amplitude 30 mV and modulation time 70 ms).

2.6. Wheat samples pretreatment

To determine OTA in the certified reference materials, aliquots of the wheat powder (10 g) were extracted with 10 mL of acetonitrile:water 6:4 (v/v) by shaking on an orbital shaker for 20 min at room temperature. Extracts were cleared by centrifugation (10 min, 3000 rpm), and 2 mL of the supernatants were mixed with 3 mL of standard solutions of OTA in PBS buffer to continue the aptasensor competitive biosensing protocol. Spiked samples of OTA (5 and 10 ng g^{-1}), used for recovering studies, were prepared 1 day before extraction by adding the appropriate volume of a methanolic OTA standard solution to 1 g of the wheat powders, followed by shaking for 20 min before extraction.

3. Results and discussion

3.1. Design strategy of OTA-responsive aptasensor assays

Common sandwich-type assays using a second biomolecule to transduce the aptamer biorecognition event (i.e. dual-site bindings) are not possible to OTA because of its small size (403.8 Da), and thus we considered competitive assays with only one aptamer capture probe (i.e. single-site binding).

In addition to magnetic separations, we first studied, optimized and compared competitive assays using disposable SPCEs. Schematic representations of these are in Fig. 1. In these preliminary studies, immobilization of the OTA–BSA (indirect assay) or the OTA aptamer (direct assay), competition, washings and detection steps were carried out completely on the SPCEs surface without using magnetic separations. Surprisingly, very few reports have been published about competitive aptamer-based assays (Centi et al., 2007; Baldrich et al., 2004), despite the advantages of needing only one aptamer-capturing probe and the reduction in the time required for the assay (only one capture incubation step). The assay procedures are shown in the supporting info appendix (section SI-1).

It was expected that the aptamer (36-mer) is only slightly perturbed upon binding with small-sized OTA, and for this reason we

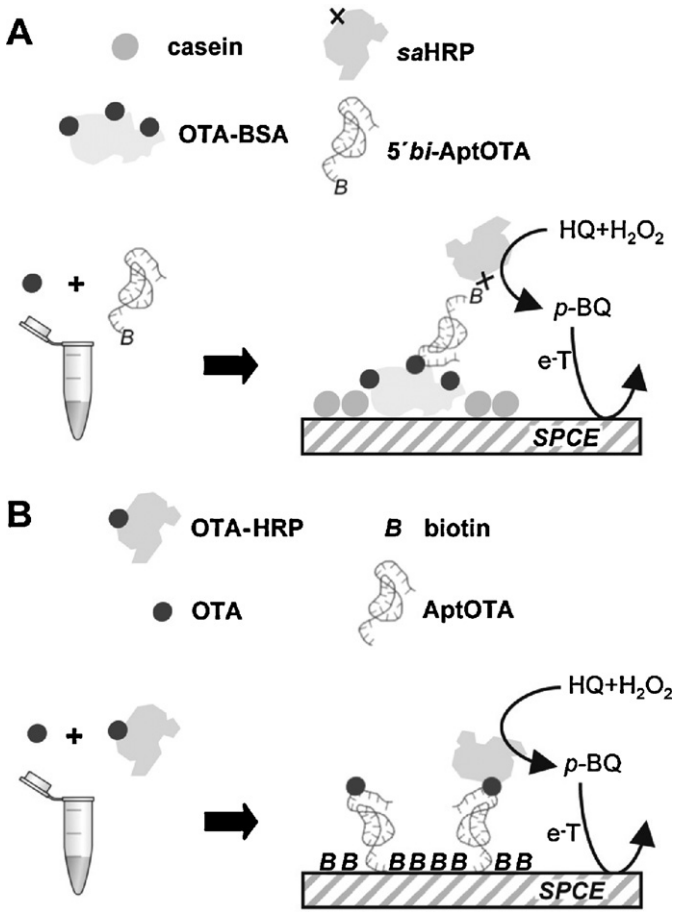

Fig. 1. Schemes of the indirect competitive assay-based aptasensor (A) and the direct competitive assay format (B) studied in preliminary tests. The immobilization, competitive and detection steps were carried out entirely on the SPCEs in both cases, without magnetic separations. X denotes streptavidin, and B biotin. The figure is not in scale.

tested competitive assays using OTA–HRP as tracer that do not depend on binding-induced conformational change in the aptamer or redox-active moieties tethered to the aptamer. Enzymatic current amplification due to the HRP enzyme was combined with these competitive aptamer-based assays, as with previous studies with OTA immunosensors carried out in our laboratory (Bonel et al., 2010).

Variables of the assays (aptamer and OTA–HRP concentrations, blocking agent, buffers, and time and temperature of the incubations) were optimized to find the best binding/assay/detection conditions (for details see section SI-2 of the supporting info). The indirect aptasensor on SPCEs (Fig. 1A) has the disadvantage of very high unspecific adsorption of the aptamer on the sensor surface from solutions, resulting in low reproducibility. With direct aptasensing (Fig. 1B), the analytical results were improved in this sense. The main problem with these two kinds of assays is the unspecific adsorption of the tracer (OTA–HRP) from solutions onto the SPCE surface, for what a very efficient blocking of the transductor electrode surface is needed, but difficult to achieve. In both formats, the competition steps of the assays (OTA and OTA–HRP binding to the aptamer sites) were carried out entirely on the electrode surface, before the voltammetric detection.

With the best conditions of the direct aptasensor (0.1 μM aptamer and 1 mg L^{-1} OTA–HRP), the inflection point (IC_{50}) was 6.87 ng mL^{-1} of OTA, and the sensitivity of the linear range of OTA concentration (from 3.03 to 8.17 ng mL^{-1} of OTA) was 0.684 ± 0.120 μA ng^{-1} mL (R = 0.9982, n = 3). The limit of detection

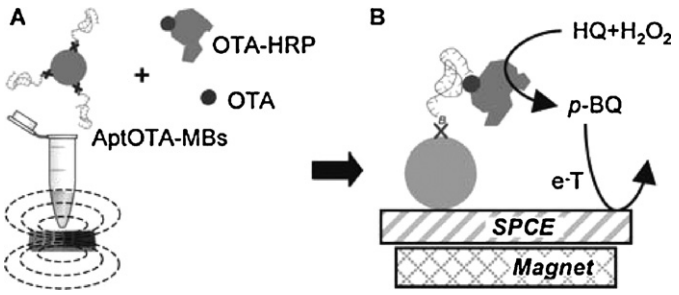

Fig. 2. Schematic representation of the magnetic aptasensor. First step: competitive and magnetic separation steps carried out in solution (A). Second step: voltammetric detection on the SPCEs under a second magnetic field (B). The scheme is not in scale.

(LOD, calculated as three times the average signal of a blank solution) was 0.77 ± 0.14 ng mL^{-1} of OTA ($n = 3$).

In an attempt to improve the sensitivity of these assays, amplified homogeneous and heterogeneous assays using gold nanoparticles functionalized with the aptamer were also tested (see the details of the method in supporting info section SI-1). We have previously found that the use of gold nanoparticles in electrochemical inmunosensors for OTA provides a stable surface for immobilization of the biorecognizing reagent and facilitates electron transfer with the working SPCE electrode materials (Vidal et al., 2009). The small size of the aptamer allow efficient immobilization at high density on the saAuNPs, but the currents obtained were too high due to very high unspecific adsorption of the OTA–HRP conjugate on the gold nanoparticles despite the use of several blocking agents (the same as used with MBs were studied). Moreover, the separation of the modified nanoparticles from solutions in the homogeneous assays did not reach 100%, with significant losses of the modified gold nanoparticles aggregates due to the inefficient separation by the centrifugation procedure.

3.2. A competitive aptamer-based biosensor for OTA using magnetic separations

The use of monosized paramagnetic beads (MBs) offers the convenience of magnetic separations, but their applications in aptamer-based biosensors is rare (Stanciu et al., 2009; Wang and Lu, 2009). Most of the reported electrochemical aptasensors coupled to magnetic beads are based on direct sandwich formats (i.e. with large molecule targets) (Centi et al., 2008, 2009a,b; Tombelli et al., 2007; Stanciu et al., 2009; Wang and Lu, 2009).

The scheme of the assay using magnetic separations is shown in Fig. 2. The competitive step was carried out separately in an eppendorf vial by mixing solutions of OTA (variable concentrations up to 1000 ng mL^{-1}), OTA–HRP tracer (a fixed optimized concentration of 1 μg mL^{-1}) and the AptOTA-MBs capturing agent, followed by incubation at room temperature (Fig. 2A). The beads modified with the aptamer (AptOTA-MBs) allow isolation and handling of target OTA in a highly specific manner. Detection of the enzymatic product was achieved by DPV in the last step of the assay, after concentrating the modified beads onto the SPCEs with the help of another magnetic field (Fig. 2B).

Typical DPV voltammograms from the optimized magnetic aptasensor with increasing concentrations of OTA are shown in Fig. 3. Higher currents are obtained with decreasing concentrations of the mycotoxin due to competitive scheme of the assay, which improves the limit of detection of the biosensor, i.e. more OTA–HRP binds to the fixed number of sites of the aptamer with decreasing OTA concentrations in the sample, obtaining higher signals. The peaks are well defined with maximum currents at a potential of about −0.125 mV corresponding to reduction of the enzymatically generated p-BQ.

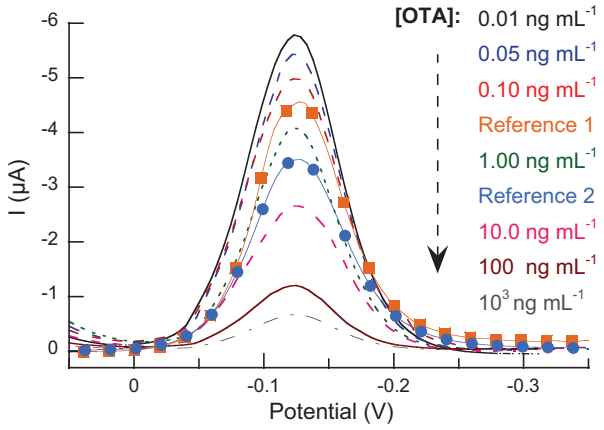

Fig. 3. DPV voltammograms of the magnetic aptasensor with increasing concentrations of OTA ranging from 0.01 to 1000 ng mL^{-1}. Marked voltammograms are from BCR-471 (reference 1, marker ■) and MYC0880 (reference 2, marker ●) wheat reference samples. Competitive OTA–HRP concentration is 1 μg mL^{-1} in all cases. Substrate of the enzyme: 5 μL of 18 mM hydroquinone and 45 μL of 1.1 mM hydrogen peroxide in HEPES buffer.

Preliminary tests in our laboratory also showed that acidic pHs of about pH = 5 notably reduce the binding efficiency of the aptamer to OTA. Under saturating conditions, we also found dependence of the aptamer folding on buffer composition: DPV peak currents increased (about 12% higher) when using 20 mM Ca^{+2} in the competition step, and thus we always used Ca^{+2} (in buffer C and D) to stabilize the structure of the aptamer to OTA complexation. Presumably, the attraction of the negatively charged OTA with the divalent cations, through the hydroxyl and carboxyl groups, favors complexation with the negatively charged aptamer. It was stated in previous reports that divalent cations influence the conformational structure of the aptamer, playing an important role in its interaction with the target molecules (Stephen and Kwong, 2007; Stellwagen and Mohanty, 2004). Accordingly, Cruz-Aguado et al. also reported that binding of OTA to the DNA aptamers is favored in the presence of divalent (Mg^{2+}, Ca^{2+}) cations (Cruz-Aguado and Penner, 2008a,b), possibly resulting from bridging-mediated interaction of the cation between the aptamer and the OTA molecule.

Unspecific adsorption of OTA-HRP (1 mg L^{-1}) on the modified beads surface was studied and quantified involving the peak height of the enzyme-generated pBQ voltammograms. Casein (1% and 5%, w/v), BSA (1% and 5%, w/v) and biotin (1000 and 10,000 mg L^{-1}) were studied as blocking agents to reduce unspecific adsorption, the best results being obtained with biotin 1000 mg L^{-1}. Increasing the length of time for the adsorption of the biotin (from 5 until 30 min) increased the blocking effect, but the opposite effect was observed with temperature (adsorption was about 1.5× greater at room temperature than at 37 °C).

To obtain the maximum sensitivity, the concentration of the aptamer bound to saMBs (0.001–10 μM) and OTA–HRP (0.01–1 μg mL^{-1}), and the incubating conditions (time and temperature) of the aptamer and the enzyme, were optimized. Crossed titration curves of OTA (from 10^{-4} to 10^{+3} ng mL^{-1}) were obtained with a fixed concentration of OTA–HRP (1 μg mL^{-1}) and different aptamer concentrations (0.001, 0.01 and 0.1 μM), and also with a fixed concentration of the 5′bi-AptOTA (0.01 μM) and several concentrations of OTA–HRP (0.1, 0.5 and 1 μg mL^{-1}). The resulting titration plots, with a sigmoidal shape typical of competitive assays, are shown in Fig. 4. The measured currents were fitted to the 4PL (four-parameter logistic) function (Bonel et al., 2010; Findlay and Dillard, 2007), and the best conditions for the assays (experimental section) were determined according to the sensitivity (inflection point, $IC_{50} = 0.52 \pm 0.04$ ng mL^{-1}, $n = 5$) and the linear range of OTA concentration (0.78–8.74 ng mL^{-1}). The slope of the linear range

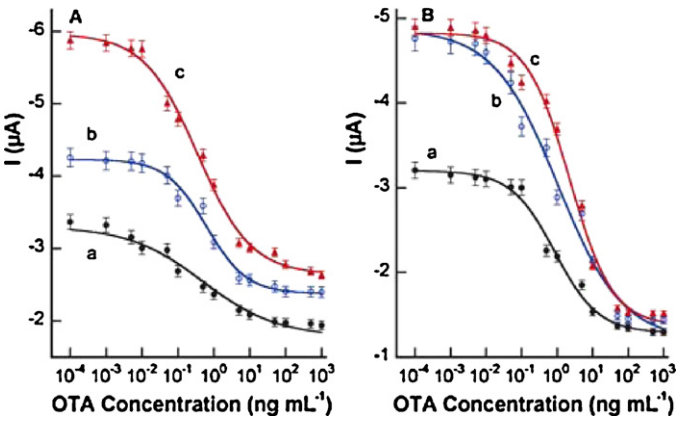

Fig. 4. Titration plots obtained during optimization of the OTA magnetic aptasensor. The immobilized aptamer (5′bi-AptOTA) concentration on the saMBs was 0.001 μM, and the three plots correspond to assays with a = 0.1 μg mL^{-1}, b = 0.5 μg mL^{-1} and c = 1 μg mL^{-1} of OTA–HRP conjugate (A). Using OTA–HRP 1 μg mL^{-1} for the competition step, the results comes from the 5′bi-AptOTA aptamer concentrations immobilized onto the saMBs of a = 0.001 μM, b = 0.1 μM and c = 1 μM concentrations (B).

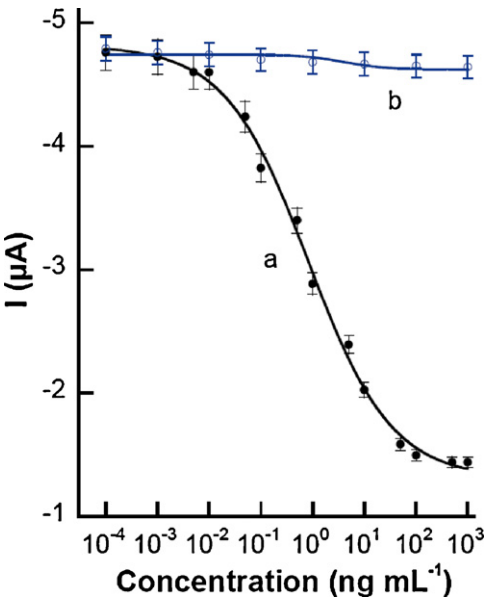

Fig. 5. Competitive titration curves for OTA (a) and OTB (b) obtained with the magnetic aptasensor using the optimized parameters (as in Fig. 2). Competitive OTA-HRP concentration is 1 μg mL^{-1} in both cases. Each point is the mean of three replicated measurements.

of OTA concentration was 1.134 ± 0.08 μA ng^{-1} mL (n = 5). Increasing the temperature from 21 to 37 °C did not improve the aptamer binding efficiency nor the kinetics of the enzymatic reaction, but immobilization of the 5′bi-AptOTA to saMBs was improved at 37 °C compared to room temperature. The limit of detection with the optimized aptasensor was 0.07 ± 0.01 ng mL^{-1} of OTA (n = 5), better than that obtained in our previous amperometric immunosensor with a polyclonal antibody (0.86 ng mL^{-1} OTA) (Bonel et al., 2010).

Magnetic separation systems allow very efficient separation and concentration of the AptOTA-MBs from buffers and other matrix components, without significant losses, and avoid unspecific adsorption of the OTA–HRP at the SPCE surface. The accessibility of the aptamer-modified MBs to diffusing target molecules favors faster assay kinetics, and with larger area, compared with the aptamer immobilized on the solid electrode surface. In addition, the magnetic field under the SPCE avoids the spread of the solutions that are precisely placed onto the surface of the electrode. So, higher currents, about 1.15×, were usually obtained compared to those obtained using the scheme of Fig. 1B when using similar concentrations of the aptamer and tracer.

The specificity of the aptamer was tested using molecular structures present in OTA. For this purpose, ochratoxin B (OTB, the dechloro-analog of OTA), warfarin (a hydrophobic molecule with a coumarin heterocycle and a phenyl group), L-Phen and HNA were studied as possible interferents.

The binding curve for OTB was obtained over the 10^{-4} to 10^{+2} M concentration range (Fig. 5) using the same methodology. OTB is a structural analog of OTA that lacks the chlorine atom in the 5-position of the isocoumarin ring. The obtained currents were in all cases very similar and corresponded to maximum values, for which the cross-reactivity of the aptameric receptor with OTB was negligible, i.e. OTB does not compete with OTA–HRP for the binding sites of the aptamer immobilized on the magnetic beads over the studied concentration range.

Concentrations of 80 ng mL^{-1} of the compounds listed were allowed to compete with OTA–HRP (1 μg mL^{-1}), in the presence of 5.5 ng mL^{-1} of OTA, and without OTA, and the electrochemical signals were compared with those obtained using blank and 5.5 ng mL^{-1} solutions of OTA. All measurements were carried out in triplicate. The DPV currents obtained were similar for all four studied interferents compared with the blank solutions (without OTA), and all of these compounds produced negligible variations in the currents of the solutions containing 5.5 ng mL^{-1} of OTA without the possible interferent.

These experiments demonstrated the high specificity to OTA of the aptamer bound to the magnetic beads, in agreement with a previous report on binding assays by equilibrium dialysis (Cruz-Aguado and Penner, 2008a,b), which found that OTB, N-acetylphenylalanine and warfarin have 100-fold less affinity to the same aptameric sequence than that of OTA.

3.3. Selective determination of OTA from wheat samples

The magnetic aptasensor was applied to wheat samples with a certified content of OTA to verify the accuracy and influence of the matrix effects. A low-content wheat certified reference material (ref. BCR-471, OTA < 0.6 μg kg^{-1} at 95% level) and wheat flour material (ref. B-MYC0880, with a certified OTA content of 2.7 ± 1 μg kg^{-1} at the 95% level) were used for spiking, recovery and accuracy studies. The analytical procedure for extracting OTA was adapted from the AOAC method 2000.3 for ochratoxin in barley (Stephen and Kwong, 2007) which is based on liquid extraction of OTA in acetonitrile:water 6:4 (v/v). It was found that small volumes of the solvent acetonitrile (approximately up to 1:10 acetonitrile:water, v/v), used for liquid extraction, do not interfere either with the affinity reaction of the aptamer with OTA or the enzymatic reaction of the peroxidase (99% of the original enzyme activity was observed with about a percentage of acetonitrile in water up to 15% (v/v)), (Bonel et al., 2010). Two typical voltammograms from the determination of OTA in reference wheat materials BCR-471 (reference 1) and B-MYC0880 (reference 2) are shown in Fig. 3.

The determination of OTA in the certified wheat materials with the magnetic aptasensor obtained average values (n = 3) of 0.47 ± 0.03 ng g^{-1} (BCR-471) and 2.67 ± 0.21 ng g^{-1} (B-MYC0880). Recovery was estimated by analyzing spiked control samples against non-extracted standards. The average recoveries of OTA ranged from about 102 ± 6% to about 104 ± 5%, with an RSD of less than about 8%, demonstrating agreement between the measured and nominal concentrations of the spiked and the certified samples.

4. Conclusions

We have demonstrated the feasibility of competitive aptasensor assay schemes for OTA coupled to paramagnetic beads and

electrochemical detection. The specificity of the DNA aptamer to OTA, enzymatic amplification, and the sensitivity of the DPV detection lead to a very specific and sensitive (down to the ng g^{-1} level) electrochemical aptasensor for the determination of OTA in wheat samples. The currents measured do not depend on conformational changes in the aptamer upon complexation with target OTA, which are usually only associated with large molecular targets such as proteins. Magnetic separations avoid unspecific adsorption of the labeled antigen (i.e. OTA–HRP) or wheat matrix on the surface of the SPCE electrodes, and allow easier access of the aptamer to the target molecule (competitive step) and higher voltammetric currents resulting from the specific location of the beads very close to the working electrode surface.

OTB did not show cross-reactivity with the biotinylated aptamer bound to the magnetic beads, and the selected aptamer bound to OTA but not to similar chemical structures like warfarin (a hydroxylated heterocycle present in many mycotoxins) or other OTA substructures (L-Phen and HNA). The sensitivity and limit of detection of the developed aptasensor is higher to that obtained using amperometric immunosensors with polyclonal antibodies to OTA or ELISAs tests, and treatment of the sample much simpler than with standard HPLC–FLD methodology. This aptasensor is very useful for the in situ determination of OTA in wheat materials well below the maximum levels allowed in the European Community for cereals (3 μg kg^{-1}) (Commission Regulation No. 1881/2006).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.12.036.

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