

Ochratoxin A nanostructured electrochemical immunosensors based on polyclonal antibodies and gold nanoparticles coupled to the antigen

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Competitive electrochemical immunosensors, based on disposable screen-printed electrodes (SPCEs), have been developed for the determination of the mycotoxin ochratoxin A (OTA). Two indirect immunoassays schemes were assessed using polyclonal antibodies and through the physical adsorption of OTA conjugated to albumin from bovine serum (OTA-BSA) and newly synthesized OTA-BSA bound to gold nanoparticles (OTA-BSA-AuNPs) onto the working electrode surface. After the competition step, detection was facilitated by a secondary *a*IgG antibody labelled with alkaline phosphatase and differential-pulse voltammetry using α -naphthyl-phosphate as substrate. The performance of the optimized immunosensors in terms of sensitivity, reproducibility and selectivity was studied. The linear working range of the described biosensors ranged between 0.9 and 9.0 ng mL⁻¹ for the OTA-BSA based immunosensor and between 0.3 and 8.5 ng mL⁻¹ for the gold nanostructured immunosensor, with a limit of detection (LOD) equal to 0.86 ng mL⁻¹ (RSD = 10.6%) and 0.20 ng mL⁻¹ (RSD = 8.0%) of OTA, respectively. The nanostructured immunosensor was applied to a certified wheat standard and a non-contaminated wheat material spiked with OTA, obtaining recoveries from about 104 ± 0.07% to 107 ± 0.08%. The influence of the wheat matrix on the analytical performance of the immunoassay was also studied.

Introduction

Mycotoxins are secondary metabolites of a range of filamentous fungi with deleterious effects for humans and animals, which can be found in agricultural commodities and animal foodstuffs.¹ They are present in trace amounts, ranging from below to few nanograms per gram of sample, and therefore, there is a need for highly sensitive and selective analytical methods for these organic toxins.² Of among all the groups of mycotoxins (aflatoxins, ochratoxins, fumonisins, zearalenone, citrinin, ergot alkaloids, *etc.*), ochratoxin A (OTA) is one of the most frequent contaminant of several foods and feeds, it is nephrotoxic and carcinogenic and poses a serious threat to the health of both humans and animals. For these reasons, the regulatory legislation of the European Community enforcing food safety is more restrictive every time.³

Instrumental methods for the analysis of OTA use mainly chromatographic based techniques,⁴ mostly based on high pressure liquid chromatography (HPLC) assisted with molecular fluorescence detection (FLD).^{5,6} A number of methods have been validated by inter-laboratory collaborative studies, many of them under the auspices of international organisms such as AOAC International.⁷ Chromatographic methods generally require multiple sample preparation steps prior to detection, including extraction, sample clean up, preconcentration and sometimes derivatization of the analyte to improve the detection sensitivity.⁸ Particularly, sample preconcentration using solid

phase extraction (SPE) and immunoaffinity columns (IAC) are very common in official methods of mycotoxin analysis using HPLC-FLD.⁹

To overcome the above limitations, a variety of immunoassay methods have been developed with isolated monoclonal and polyclonal antibodies raised against the specific mycotoxins,^{10–12} particularly for OTA.^{13–15} The very selective immunological basis of such methods makes them highly specific and, therefore, less dependent on sample cleanup. So far, enzyme-linked immunosorbent assay (ELISA) is the most common immunoassay technique used in OTA analysis due to simplicity and capability for parallel analysis of multiple samples.^{13,16,17}

A number of ELISA test kits have also been commercially developed for many important mycotoxins, including OTA, which are useful tools for screening and quantification at low ppb levels¹⁸ and may be used for *in situ* measurements of mycotoxin occurrences. They have benefits in speed and sensitivity, but might have some drawbacks such as cross reactivity and matrix dependence which would result in over-estimation of the analyte concentration (possibility of false-positive results).^{16,19}

More recently, the use of biosensors allows a major portability, *in situ* analysis of OTA with similar selectivity and sensitivity to ELISAs, simultaneously with minor consumption of reagents and much shorter times of analyses.^{20–23} They are mostly based on immunological principles (immunosensors), and in many recent years have also appeared devices based on specific aptamers against OTA (aptasensors).²⁴

Amperometric immunosensors that use antibodies (or antigens) attached to the surface of electrodes are especially suitable in this sense,²⁵ particularly using disposable screen-printed electrodes (SPCEs).²⁶ Direct or indirect immobilizations onto the SPCEs

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are based on distinct functional reagents such as diazonium organic salts²⁵ or biotin/avidin chemistries,²⁷ or by simple physical adsorption of the proteins. Limits of detection of immunosensors on SPCEs lie in the order of 0.5–20 ng mL⁻¹ and dynamic ranges up to concentrations of about 1–100 ng mL⁻¹ of the mycotoxins. For instance, a surface-adsorbed antibody against aflatoxin B1 (AFB 1) gives a detection limit of around 0.15 ng mL⁻¹ in buffer solution.²⁸ Nitrocellulose membrane-based ELISA immunoassays have also been described for the simultaneous screening of aflatoxin B1 and OTA in chilli samples, with limits of quantitation of 2 and 10 ng g⁻¹ respectively.²⁹ Palleschi *et al.* have described a monoclonal antibody based electrochemical immunosensor for the determination of OTA, with limits of detection in the range 60 to 100 µg L⁻¹ in direct and indirect assay formats, although the synthesis of an OTA-alkaline phosphatase conjugate is necessary for revealing the signals of the biosensors.¹⁵

On the other hand, metallic nanoparticles, in particular gold nanoparticles (AuNPs), are nowadays of great interest in new generation of bioelectronic devices with increased sensitivity, high biocompatibility and novel functions.³⁰ Particularly, some different nanostructured surfaces on SPCEs immunosensors have been proposed, like electrochemical generated AuNPs from AuCl₄⁻,³¹ adsorbed AuNPs stabilized by the synthetic membrane-like compound didodecyl-dimethyl-ammonium bromide,³² labelling of secondary antibodies,^{33,34} localization of colloidal gold-labeled protein A on the antigen-antibody immune complex,³⁵ stabilizing the gold colloid with BSA and glutaraldehyde,³⁶ PAH (poly allylamine hydrochloride)-modified, nanosized-Au particles assemblies,³⁷ synthesis of the nanoparticles on conducting polymers³⁸ or drop-coating AuNPs onto polythionine modified electrode surface for the determination of aflatoxin B1.³⁹

In a previous paper, we have characterized kinetics and affinity of polyclonal antibodies against OTA by measuring small mass changes with a quartz-crystal microbalance (QCM),⁴⁰ which was applied to QCM immunosensors for the mycotoxin. Covalent attachment of the OTA-BSA conjugates through gold nanoparticles produced mass-amplified signals, but this enhancement did not reach to detect some of the required very small concentrations of OTA necessary for certain kind of samples.

In this paper, indirect competitive immunosensors for OTA are developed with the same polyclonal antibodies, but using disposable screen-printed electrodes and differential-pulse voltammetry. Additionally, improvements of nanostructured immunosensors with newly synthesized molecules of the antigen bound to gold nanoparticles are also studied. Sensitivity was greatly improved with regard to our previously developed QCM immunosensors for OTA.⁴⁰

Experimental

Materials and equipment

Screen-printed electrodes (SPCEs) were made in-house in a multistage deposition process onto 1 mm thick substrate of flexible fr-4 epoxy resin sheets. Graphite based ink (Electra ED7100) was used for the working electrode. A carbon based counter electrode and a silver pseudo-reference electrode were also

printed onto the same substrate. In the final step, a layer of insulating ink was screen printed over the electrodes to give the disk shaped exposed working electrode ($\varnothing = 4$ mm, 0.126 cm²). The intra batch variation of ten lots of ten electrodes, calculated *via* the chronoamperometric determination of the bare working area of each electrode in 80 µL of a solution of α -naftol 10 mg L⁻¹, was observed to be less than 10% ($n = 10$), indicating good reproducibility. The printed foils were stored dry, at room temperature, in the dark. Before using, the screen printed electrodes surface were electrochemically pretreated in 70 µL of 0.2 M H₂SO₄ with three cyclic voltammetry potential scans carried out between -1.2 V and +1.5 V at a scan rate of 100 mV s⁻¹.

All electrochemical measurements were performed on an Eco Chemie potentiostat model PGSTAT 1212 (Utrecht, Netherlands). The immunosensor measurements were carried out at room temperature by using differential-pulse voltammetry (DPV) with the following parameters: range potential from 0 to +600 mV (1-naphthol), or from 0 to -800 mV (for p-benzoquinone formed by HRP), step potential 2 mV, scan rate 10 mV s⁻¹, modulation amplitude 30 mV and modulation time 70 ms.

Chemicals

Ochratoxin A (OTA) from *Aspergillus Ochraceus* (*Aspergillus Oryzae*) ($\geq 98\%$) was obtained from Scharlab (Barcelona, Spain). Aliquots from a stock solution of OTA (100 µg mL⁻¹, 10 mL) were stored at -20 °C, and diluted at room temperature with PBST buffer to the desired concentrations when required. Bovine serum albumin ($>98\%$) (BSA), casein from bovine milk, gold colloidal nanoparticles (20 nm diameter, $\sim 0.01\%$ HAuCl₄, ref. G1652), ochratoxin A conjugated to BSA (OTA-BSA, about 3.8 molecules of OTA in one molecule of BSA), anti-rabbit IgG-Peroxidase (*a*IgG-HRP, 1.0 mg mL⁻¹), anti-rabbit IgG-Alkaline Phosphatase (*a*IgG-AP, 1.1 mg mL⁻¹), α -naphthyl phosphate disodium salt, hydroquinone ($\geq 99\%$), and hydrogen peroxide (30%) were purchased from Sigma-Aldrich (Madrid, Spain). Polyclonal rabbit antibody against *Aspergillus Ochraceus* OTA (*p*AbOTA, 1 mg mL⁻¹) was from AbD Serotec (Madrid, Spain). Other analytical reagents were from Merck (Darmstadt, Germany). Wheat certified reference material BCR-471 (OTA < 0.6 µg Kg⁻¹ at 95% level) was from IRMM (European Commission) and certified by BCR. OTA in wheat flour material (ref. B-MYC0880, lot#M07453Z), with a certified mass concentration of 2.7 ± 1 µg Kg⁻¹ at a 95% level, was from Biopure (Tulin, Austria).

Immunosensor assays with *p*AbOTA

All steps of the immunoassays and electrochemical measurements were carried out onto the surface of the SPCEs. In between the sequential steps (coating, binding, competition and revealing) the surface of the electrodes were carefully washed by duplicate with PBST buffer (0.1 M phosphate buffer + 0.138 M NaCl + 0.05% Tween-20, pH 7.4). First, 10 µL of 3 µg mL⁻¹ of the OTA-BSA conjugate in CB buffer (0.05 M carbonate, pH = 9.6) was deposited onto the working electrode of the SPCEs and left to adsorb for 20 h at 4 °C. Then 10 µL of a blocking solution of 1% (w/v) casein in PBST buffer was added and left at 37 °C for 30 min. Competitive tests were made with addition of 10 µL of

pre-mixed solutions (they were vortexed for about 10 s in eppendorf vials) made with 10 μL of OTA standards in PBS buffer (0–1000 $\mu\text{g L}^{-1}$) or sample, and 10 μL of *pAbOTA* antibody (3 mg L^{-1}), following incubation during 30 min at 37 $^{\circ}\text{C}$. After washing, 10 μL of the secondary antibody *aIgG-AP* (1.1 mg L^{-1} , dilution 1 : 1000) in PBS containing 1% casein was left to react for 30 min at 37 $^{\circ}\text{C}$. At last, 60 μL of a solution α -naphthyl phosphate 1 mg mL^{-1} in buffer DEA (0.1 M diethanolamine buffer + 1 mM MgCl_2 + 100 mM KCl + 1% casein, pH 9.6) was deposited onto the screen-printed and after 5 min the product of the enzymatic reaction (α -naphthol) was determined by DPV.

Synthesis of OTA-BSA-AuNPs conjugate

The OTA-BSA-colloidal gold conjugate (OTA-BSA-AuNPs) was prepared by adding 48.5 μL of 1 mg mL^{-1} of OTA-BSA conjugate to 1 mL colloid gold solution ($\sim 0.01\%$ HAuCl_4) while gently stirring and adjusting to pH = 6.0 with 0.1 M NaOH. The mixture was incubated at room temperature for 1 h, then 200 μL of PBS with 5% BSA was added into the mixture to stabilize the colloidal gold solution and the resultant was additionally incubated for 30 min. Upon centrifugation at 13400 rpm for 10 min, two phases were obtained: a clear to pink supernatant of unbound OTA-BSA and a dark red sediment of conjugate. The supernatant was discarded and the sediment was rinsed by re-suspending in 1 mL of PBS with 0.1% BSA and collected after a second centrifugation (13400 rpm) for 10 min. At last, the conjugate was re-suspended in 100 μL of tris-buffered saline buffer (20 mM tris + 150 mM NaCl, pH 8.0 adjusted with HCl), containing 0.1% BSA added to minimize nonspecific adsorption and increase the stability of the conjugate during the immunoassays. The nanoparticle conjugates were stored at 4 $^{\circ}\text{C}$ for more than 1 month without significant decrease in activity.

Immunosensor assays with polyclonal antibodies and gold nanoparticles

The immunosensor assay procedure is similar to that explained before but with slight changes, and it was also based on an indirect competitive format. The immobilization of the antigen was performed in this case with 10 μL of AuNPs-BSA-OTA conjugate (2.5 mg L^{-1}) in 0.5 M CB buffer (0.05 M carbonate, pH = 9.6) and kept overnight at 4 $^{\circ}\text{C}$. An incubation temperature of 4 $^{\circ}\text{C}$ was applied to diminish the effect of possible nonspecific interactions that is more likely to arise with higher temperatures. The binding or competition studies were carried out by adding 10 μL of *pAbOTA* solutions or 10 μL of pre-mixed solutions of OTA + *pAbOTA* respectively, following incubation at 37 $^{\circ}\text{C}$ during 30 min. The competitive solutions were prepared in eppendorf vials by mixing (vortexing for about 10 s) 10 μL of OTA standard solutions (0–1000 $\mu\text{g L}^{-1}$ in PBS buffer) or sample, with 10 μL of *pAbOTA* antibody (5 $\mu\text{g mL}^{-1}$), and they were dispensed onto the SPCEs in about 10 s. All the other blocking, washing and revealing steps were as explained before.

Analysis of wheat extracts

Calibration curves for OTA were produced by spiking the non-contaminated wheat extracts with OTA standard solutions. For

this purpose, aliquots of the non-contaminated wheat powder (1 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 g), and 1 mL of the supernatants was mixed with 4 mL of OTA standards in PBST buffer for detection by the immunosensor.

To determine the OTA in the certified material, 1 g of the powder sample (or non-contaminated wheat spiked with OTA standards) was extracted with 10 mL of the extraction solvent (acetonitrile : water 6 : 4 (v/v)), mixed in a rotating stirrer for 5 min and centrifuged at 3000 rpm for 10 min, 1 mL of the supernatant was then diluted in 4 mL of PBST buffer and used for the detection of OTA. Spiked samples of OTA (5 and 10 ng g^{-1}), used recovery studies, were prepared 1 day prior to extraction by adding the appropriate volume of a methanolic OTA standard solution to 5 g of the non-contaminated wheat powder and were shaken for 20 min before extraction. The concentrations of OTA in wheat extracts were determined from the calibration curve and it was used to calculate the concentration of the original samples.

Results and discussion

Immunosensor assays using polyclonal antibodies: optimization of the experimental factors

The main objectives of this work are the development of optimized *pAbOTA* immunosensors for detecting OTA and the improvement of the analytical sensitivity due to nanostructuring using gold nanoparticles (AuNPs). Sandwich immunoassay schemes are not possible with OTA due to its small molecular weight, and only indirect competitive immunoassays were considered.

The immunoassay schemes used are depicted in Fig. 1 and they were based on immobilization of OTA-BSA (Fig. 1A) or OTA-BSA-AuNPs (Fig. 1B) conjugates onto the SPCEs. Calibration and binding curves were obtained entirely onto the modified SPCE surface by adding a fixed quantity of antibody (*pAbOTA*) which competes with the immobilized antigen and OTA in solution, and the amperometric currents were revealed through secondary antibodies labelled with alkaline-phosphatase (*aIgG-AP*).

Checkboard titrations of OTA-BSA-AuNPs, *pAbOTA* and secondary *aIgG-AP* concentrations were optimized, as in

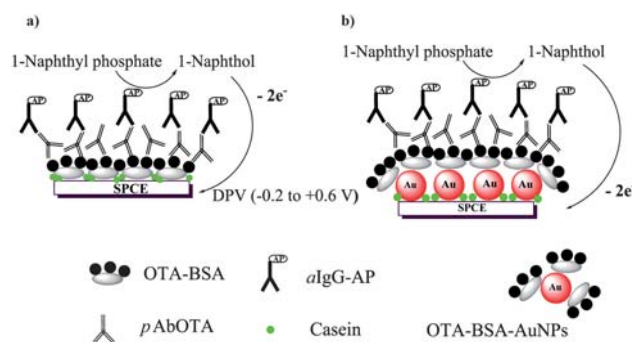


Fig. 1 Scheme of the two immunosensor designs: a) coating with OTA-BSA onto the working surface electrode; b) nanostructuring by coating with OTA-BSA-AuNPs.

a conventional ELISA spectrophotometric assay, to obtain the maximum electrochemical sensitivity. This optimisation was of paramount importance since the behaviour of antibodies fixed onto the SPCEs solid supports (in heterogeneous phase) can vary considerably with respect to their performances in solution. After characterization of the affinity through these binding curves, competitive conditions between immobilised OTA, *pAbOTA* and free OTA in solution were also optimized.

The unspecific adsorption of the primary and secondary antibodies on the carbon surface of the SPCEs can seriously affect the performance of the immunosensor protocols, for what a blocking agent is necessary. For this purpose, additions of 10 μL of BSA and casein proteins 0.01%, 0.1% and 1% (w/v) in PBS were compared as blocking agents. Measurements with bare and blocked SPCEs after incubating solutions of *pAbOTA* (1 : 1000)+*aIgG-AP* (1 : 1000), without antigen, following the electrochemical procedure, showed a significant decreasing of the currents with the protein modified electrodes. Better results were obtained for casein 1% (w/v) (blocking percentage about 92%) than with BSA 1% (w/v) (about 78%).

The sensitivities of two secondary *aIgG-AP* and *aIgG-HRP* antibodies, necessary for revealing the extent of reaction of the *pAbOTA*, were compared. The working electrode surfaces were modified with a fixed addition of 10 μL of 2.5 $\mu\text{g mL}^{-1}$ of OTA-BSA, saturated with 10 μL of *pAbOTA* 5 $\mu\text{g mL}^{-1}$ and blocked with 10 μL of casein 1% (w/v). Then, 10 μL of the secondary antibody solutions (in a range of concentrations 0–100 mg L^{-1}) were added and the final currents measured after following the immunoassay protocol. The currents obtained are plotted in Fig. 2. The substrate solution used for HRP was 5 μL of 18 mM hydroquinone and 45 μL of the co-substrate 1.1 mM hydrogen peroxide in PBS buffer (0.1 M PBS + 1% BSA, pH = 7.4). The background currents measured were similar in both cases (indicating similar non-specific adsorption), whereas

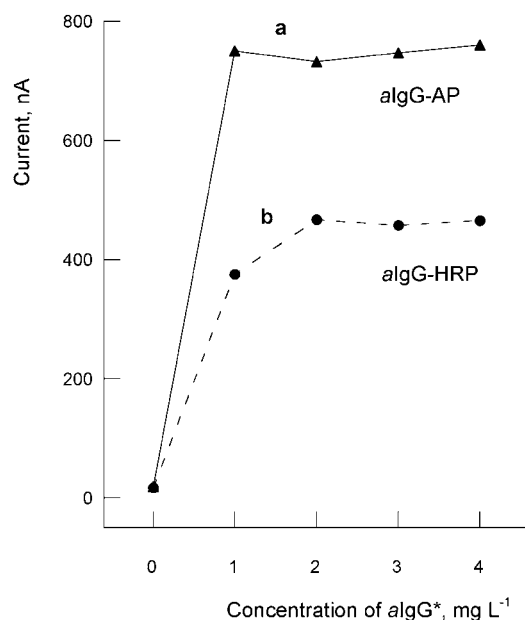


Fig. 2 Influence of the enzyme label and concentration of the secondary antibody on the measured currents: a) *aIgG-AP* (10 μL); b) *aIgG-HRP* (10 μL). Data in *x*-axis are not in scale.

the maximum currents measured with the secondary antibody are higher with *aIgG-AP* (about 755 ± 5.1 nA) than with *aIgG-HRP* (about 455 ± 9.2 nA). Higher amounts of 1 mg L^{-1} of the secondary antibodies did not increase the currents in both cases (Fig. 2). We decided to use in further studies the *aIgG* labelled with AP for the voltammetric detection due to their best sensitivity with respect to the HRP labelled antibody. From a number of substrates that can be used as electrochemical mediator for alkaline-phosphatase (AP), we decided to use α -naphthyl phosphate as substrate because of the good redox properties of the α -naphthol, which is oxidised under irreversible diffusion-controlled conditions at the SPCEs.

The currents obtained did not increase with incubation times between OTA-BSA and *pAbOTA* greater than 30 min at 37 $^{\circ}\text{C}$ (times from 15 to 60 min were studied). Neither the signals increased with reaction times between *pAbOTA* and *aIgG-AP* longer than approximately 25 min at the same temperature. The highest currents are measured with incubation times between the substrate (α -naphthyl phosphate) and the enzyme (AP) of 6–9 min. Nevertheless, the current increases for more than 5 min are very small (about 50.6 ± 2.3 nA), so we used this time. In this way, the assay is quicker and contributes to obtain a less time consuming device, which is very important in the development of commercial immunosensors.

For the best competitive conditions, binding curves of dilutions of *pAbOTA* (1/10 to 1/1000) with different previously coated OTA-BSA antigen concentrations (1 to 5 $\mu\text{g mL}^{-1}$) were obtained following the indicated procedure and with *aIgG-AP* 1.1 mg L^{-1} (1/1000 dilution). The *pAbOTA* quantity gives the excess with respect to analyte that competes with the sites of the immobilized antigen. The optimum dilutions of the *pAbOTA* (at different coated antibody concentrations) are a compromise between the maximum difference of the currents (bound-unbound) and the inflection points of the titration curves (EC_{50}). The current asymptote differences (at zero and maximum analyte concentrations) at the binding curve increases were very small when increasing the antibody concentrations (about $15\text{--}25 \pm 0.36$ nA, possibly due to saturation of all accessible sites of the immobilized antigen reacted), and only a much higher antibody concentration produced a slight decrease of EC_{50} . A *pAbOTA* concentration equal to 3 mg L^{-1} was chosen as optimum.

Competitive calibration curves using standard solutions of OTA (from 0 to 1000 ng mL^{-1}) under the optimized conditions are shown in Fig. 3a. The currents obtained by DPV measurements increase with a decrease of OTA concentrations, a typical behaviour of an indirect competitive immunoassay. Three replicates were made of each point of the calibration. The standard curves were fitted by nonlinear regression using the 4-parameters logistic (4-PL) equation:⁴¹

$$f(x) = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + (x/\text{inflt})^{\text{slope}}}$$

in which *top* and *bottom* are the asymptotic maximum and minimum currents, *slope* is the slope of the linear part, and *inflt* is the OTA concentration (*x*) necessary to half the current signal (inflection point EC_{50}) of the curve. Results of the standard curve parameters are summarized in Table 1, and the calculated analytical performances of the immunosensors are in Table 2.

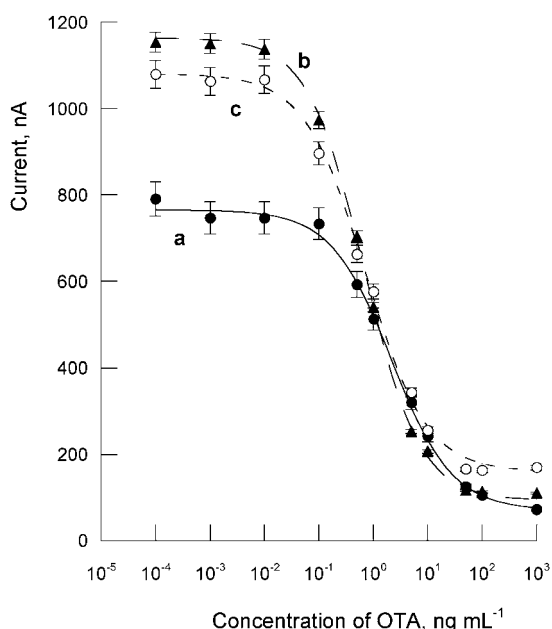


Fig. 3 Standard calibration plots obtained for OTA standard solutions (0–1000 ng mL⁻¹) in PBS using the SPCEs immunosensors: a) Coating with OTA-BSA (10 μ L, 2.5 μ g mL⁻¹); b) Coating with conjugated antigen bound to nanoparticles (OTA-BSA-AuNPs) (10 μ L, 3 μ g mL⁻¹); c) Same as in b) but spiking with OTA non-contaminated wheat-extracts. Error bars represents the relative deviation of the three measurements of each point (n = 3).

Table 1 Competitive data of the immunosensors using the four parameter logistic (4-PL) curve fitting

	top	bottom	slope	EC ₅₀	4-PL regression
OTA-BSA ^a	765.3	70.5	0.780	2.17	0.9986
OTA-BSA-AuNPs ^a	1163	93.5	0.843	0.68	0.9989
OTA-BSA-AuNPs ^b	1080	160	0.788	0.72	0.9979

^a Competition of OTA in PBST buffer. ^b Competition of OTA in wheat extracts.

Table 2 Analytical performance of the immunosensors in PBS and in wheat extracts

	LOD ^{a/} ng mL ⁻¹	EC ₅₀ ^{b/} ng mL ⁻¹	Linear Range/ng mL ⁻¹	CV/(%)
OTA-BSA ^c	0.86	2.17	0.9–9.0	10.6%
OTA- BSA-AuNPs ^c	0.20	0.68	0.3–8.5	8.0%
OTA- BSA-AuNPs ^d	0.21	0.72	0.4–7.2	7.8%

^a LOD, limit of detection = (blank – 3 sd) of the zero point.

^b Concentration of OTA at 50% inhibition. ^c Calibration curves for OTA in PBST buffer. ^d Calibration curve in “blank” wheat extracts.

Sensitivity of the OTA-BSA immunosensor was high (EC₅₀ = 2.17 ng mL⁻¹ OTA). The linear concentrations ranged from 0.9 to 9.0 ng mL⁻¹, with a detection limit of 0.86 ng mL⁻¹. The intra-electrode reproducibility was 10.6% rsd (n = 3 replicates) and the inter-assay repeatability about 15% rsd (n = 3). Once the antigen is immobilized on the SPCEs, the time consumed for the immunosensor measurements is about 2 h.

Immunosensor assays using antigen-gold nanoparticles nanostructuration

The use of AuNPs was considered with the purpose of increasing the quantity and accessibility of the immobilized OTA, besides an improvement of the amperometric currents due to dispersed metallic particles at the nanoscale. For this purpose, the OTA conjugated to BSA was covalently bound to the gold nanoparticles before being immobilized onto the working electrode surface. The competitive immunoassay scheme is similar to the previous one (Fig. 1B), but the presence of the NPs produced slight changes in the optimum concentrations of the immunoreagents. It was expected that nanostructuration with 20 nm gold particles would increase the accessibility of the conjugated OTA with higher number of effective antigen sites on the same surface of the working electrode, besides an increase of the charge-transfer kinetics in voltammetric measurements.

As before, binding curves of *pAbOTA* (from 0 to 10 μ g mL⁻¹) at different previously coated (incubated overnight at 4 °C) OTA-BSA-AuNPs (from 0 to 4 μ g mL⁻¹), using a fixed concentration of *aIgG-AP* (1.1 μ g mL⁻¹, dilution 1 : 1000) were compared to assess optimal concentrations of the immunoassays. The saturation of the working electrode with OTA-BSA-AuNPs was obtained by coating with 10 μ L of 2.5 μ g mL⁻¹ concentration, which were reacted using an excess of the antibody (*pAbOTA*, 10 μ L, 8 μ g mL⁻¹). Maximum currents were about 1160 \pm 72.8 nA (CV = 6%) at these conditions (higher than with immunosensor without nanoparticles), which signifies an increased sensitivity (due to nanoparticles) and also the maximum accessible sites of the antigen (more concentration of immobilized OTA-BSA-AuNPs did not increase signals). The optimal concentration of *pAbOTA* determined at about EC₅₀ of the binding curves (defined as 50% of the inhibition concentration) for making a competitive assay was 5 μ g mL⁻¹.

Without *pAbOTA* in the competition step, the currents measured were similar with and without the antigen. The low measured currents (80 \pm 6.6 nA) in such conditions are correlated with a low non-specific binding of the secondary antibody.

The competitive binding curve for OTA standard solutions with *pAbOTA* (5 μ g mL⁻¹) is plotted in Fig. 3, the corresponding mathematical data (using the four-parameter logistic equation) are in Table 1, and the analytical performance of this immunosensor is summarized in Table 2. As the reproducibility of the AuNPs immunosensor (OTA-BSA-AuNPs) was better than with the first one (OTA-BSA), especially with the highest currents at zero OTA concentrations, LOD was lower due to nanostructuration (1.5 ng mL⁻¹, Table 2). The sensitivity was also better with the OTA-BSA-AuNPs as the smaller value of EC₅₀ (0.68 ng mL⁻¹ OTA), and the slope of the linear part is also higher, as it can be seen in Tables 1 and 2. The linear range is slightly broader (0.3 to 8.5 ng mL⁻¹ of OTA) with this nanostructured biosensor.

Determination of OTA in wheat samples

The more sensitive nanostructured immunosensor was applied to wheat extracts. The principal intention of the immunosensors is to minimize the matrix effects of real samples, owing to the selectivity of the appropriately selected primary antibody. The

sample preparation procedures should also minimize matrix interferences and inhibition effects of the immunoassays.

At first, evaluation of the matrix effect in immunoassays was carried out, and secondly the study of the extraction efficiency of OTA from contaminated (or spiked) wheat samples to verify the accuracy of the biosensors. For this purpose, we used two sample materials: a non-contaminated wheat powder with a certified content of OTA $<0.6 \mu\text{g Kg}^{-1}$ (95% level) and a contaminated wheat flour powder with an OTA content of $2.7 \pm 1 \mu\text{g Kg}^{-1}$ (certified at a 95% level).

As said, a variety of food commodities are subjected of OTA contamination, the most important cereals and cereal products. Conventional analysis of solid samples usually begins with extraction in organic (*e.g.* chloroform, hexane-acetonitrile, dichloromethane-ethanol, or ethyl acetate) or in aqueous-organic solvents (*e.g.* methanol-water, acetonitrile-water), containing additives like sodium hydrogen carbonate or magnesium chloride to enhance solubility and extraction efficiency of OTA. We used an extraction procedure to liberate OTA from the samples which was based on the AOAC official method 2000.3 for ochratoxin in barley,⁸ with minor changes, extracting the analyte by blending with acetonitrile-water (6 : 4 v/v) as explained.

To evaluate the matrix effect on the OTA-*pAbOTA* interaction, binding curves were carried out by preparing different dilutions of OTA in extracts of non-contaminated wheat. The phosphatase alkaline activity was not affected by small amounts of acetonitrile used for extraction, in accordance with other authors,¹⁵ but sensitivity changes were produced by the wheat extracts matrix (Table 1). The binding curve of the spiked wheat extracts (Fig. 3 and data of Table 2) showed minor differences between the currents at the top and the maximum (*i.e.* the currents of the ends of the 0 and maximum competition) with respect to that obtained without the matrix of the wheat-extracts (Fig. 3), as well as a decreased slope of the linear part of the graph, which would signify the partial inhibition of the OTA with *pAbOTA* reaction. Nevertheless, because the value of EC_{50} is only slightly higher due to wheat matrix (0.72 against 0.68, Table 1), the immunosensor allows the determination of concentrations of OTA with similar sensibility. The detection limit for wheat sample extracts was 0.72 ng mL^{-1} of OTA (Table 2), based on a signal to noise ratio of 3 : 1.

To verify the accuracy of the biosensors, the certified and spiked with OTA wheat samples were extracted and analysed for recovery calculations using the calibration curves of the matrix extracts. Recovery was estimated by analysing spiked control samples against non extracted standards. The recovery

experiments were made by duplicate, and the OTA concentration was calculated by interpolation in the 4-PL calibrating equation of the “blank” extracts. The results are summarized in Table 3. The average recoveries of OTA ranged from about $104 \pm 7\%$ to about $107 \pm 7.9\%$, with RSD lower than about 8%, which show the agreement between measured and nominal concentrations of the spiked and the certified samples.

Conclusions

There is an important need for fast, easy-to-use devices for detection of mycotoxins in complex matrixes, such as cereals, wine, or beer. In this context, disposable biosensors (SPCEs) capable of measuring *in situ* very few ppbs of OTA, with rapidity and small instrumentation, suppose a big advance in mycotoxin analysis. Voltammetric immunosensors combine the high selectivity of the immunoanalysis with the ease and sensitivity of the electrochemical detection. We developed and compared analytical properties of two OTA-BSA and OTA-BSA-AuNPs indirect competitive immunosensors onto disposable SPCEs, of which the second nanostructured biosensor reaches more sensitivity ($EC_{50} = 0.68 \text{ ng mL}^{-1}$) and lower LOD (1.5 ng mL^{-1} of OTA) due to more accessible sites of the immobilized antigen on the surface of the electrode and increased electrocatalyzed currents due to gold nanoparticles. The parameters that can influence the immunoassay procedure coupled to the affinity reaction of OTA with *pAbOTA* were previously optimised using the SPCEs by electrochemical measurements using alkalino-phosphatase labelling (more sensitive than DPV measurements with HRP enzyme). These immunosensors are capable of measuring OTA in wheat samples with a linear range of 0.4 to 7.2 ng g^{-1} of OTA, with a limit of detection of 0.21 ng g^{-1} OTA, and with recoveries of OTA from extracted wheat samples ranged from about $104 \pm 7\%$ to $107 \pm 7.9\%$. The extraction procedure and the nanostructured immunosensor allow the determination of OTA below EU fixed regulatory limits for cereals.

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Table 3 Recovery of OTA from extracted spiked wheat samples and results of the analysis of the certified reference sample. Results are the mean of three analysis

	Added ^a	Found ^a	RSD	Overall mean recovery, %
Spiked 1	5	5.2	7.2	104.4
Spiked 2	10	10.4	7.6	103.9
Certified B-MYC880	2.7 ± 1^b	2.9	8.0	107.4

^a Units: ng g^{-1} . ^b Certified mass concentration.

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