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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).