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An aptasensor for ochratoxin A based on grafting of polyethylene glycol on a boron-doped diamond microcell

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ABSTRACT

A novel strategy for the fabrication of an electrochemical label-free aptasensor for small-size molecules is proposed and demonstrated as an aptasensor for ochratoxin A (OTA). A long spacer chain of polyethylene glycol (PEG) was immobilized on a boron-doped diamond (BDD) microcell via electrochemical oxidation of its terminal amino groups. The amino-aptamer was then covalently linked to the carboxyl end of the immobilized PEG as a two-piece macromolecule, autoassembled at the BDD surface, forming a dense layer. Due to a change in conformation of the aptamer on the target analyte binding, a decrease of the electron transfer rate of the redox $[Fe(CN)_6]^{4-/3-}$ probe was observed. To quantify the amount of OTA, the decrease of the square wave voltammetry (SWV) peak maximum of this probe was monitored. The plot of the peak maximum against the logarithm of OTA concentration was linear along the range from 0.01 to 13.2 ng/L, with a detection limit of 0.01 ng/L. This concept was validated on spiked real samples of rice.

Ochratoxins are a group of mycotoxins produced by several fungal species of the genera *Aspergillus* and *Penicillium* [1]. Ochratoxin A (OTA, *N*-[(3*R*)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl) carbonyl]-L-phenylalanine, MW = 403.81 g/mol) is the most prevalent of this group and is considered as one of the most important toxic fungal secondary metabolites in food safety regulation. Although it has been widely studied since its discovery in 1965 [2], public awareness of this mycotoxin has recently increased [3]. Extensive toxicity studies suggest that OTA is immunosuppressive [4] and teratogenic [5] and that it impairs blood coagulation [6] and affects glucose metabolism [7]. OTA is known for its nephrotoxicity in all animal species tested [8], and it has been classified as a possible human carcinogen [9]. Several food products, such as beer, wine, nuts, liquor, rice, coffee, cocoa, meat, and spices, are susceptible to OTA contamination [2].

Typical OTA detection methods make use of chromatographic techniques. Notably, high-performance liquid chromatography—fluorescence detection [10] has been adopted as an official method. However, significant resources and efforts have been devoted to modifying these techniques and to developing new detection methods for improved cost, speed, and detection sensitivity. Sensing platforms, such as capillary electrophoresis, solid-phase extraction—liquid chromatography—electrospray tandem mass spectrometry, and solid-phase microextraction—liquid chromatography—fluorescence detection, have been developed. In many of these detection methods, a toxin-specific antibody is frequently applied as a molecular recognition agent [11].

Aptamers are single-stranded nucleic acid ligands that are isolated from random-sequence DNA pools through an in vitro selection process known as SELEX (systematic evolution of ligands by exponential enrichment) [12]. Aptamers are capable of binding tightly and specifically to targets ranging in size from small organic molecules, proteins, and even to cells. Moreover, aptamers offer many advantages when compared with antibodies, which are biologically produced antigen-specific proteins. Antibodies cannot be easily obtained for small-size targets (e.g., metal ions) or for molecules with poor immunogenicity or high toxicity, although it

Abbreviations: OTA, ochratoxin A; SELEX, systematic evolution of ligands by exponential enrichment; BDD, boron-doped diamond; LOD, limit of detection; PEG, polyethylene glycol; SPCE, screen-printed carbon electrode; PEG-Apt, PEG aptamer; NHS, *N*-hydroxysuccinimide; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride; OTB, ochratoxin B; CV, cyclic voltammetry; SWV, square wave voltammetry; AFM, atomic force microscopy; CPA, carboxy-PEG[77]-amine; EIS, electrochemical impedance spectroscopy; AFB1, aflatoxin B1; AFM1, aflatoxin M1; RSD, relative standard deviation; PBS, phosphate-buffered saline.

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may be possible to design aptamers for such target analytes. Besides, aptamers can be chemically modified very easily, and this permits them to be immobilized over a wide range of transducer surfaces [13].

A wide variety of electrodes have been used as a support to fabricate electrochemical aptasensor devices. These include carbon paste, glassy carbon, gold, and screen-printed electrodes. Therefore, boron-doped diamond (BDD) electrodes in a single microcell were chosen to fulfill these requirements in the current work. BDD electrodes have received increasing attention for both kinetics studies of the electrode process and quantitative analyses. As is well known, BDD electrodes have the advantages of low background current, chemical and mechanical stability, resistance to fouling, a wide potential window, poor adsorption of polar molecules, and controllable surface termination [14]. BDD electrodes can enhance the limit of detection (LOD), the high oxygen evolution potential, and the negative hydrogen evolution potential, and they can allow improved direct surface electrochemical modification.

Most aptamers are known from previous research studies, and their unique three-dimensional conformation that occurs on target binding is well known. This conformational alteration characteristic of aptamers facilitates and enhances the detection phenomena of small-size target analytes by enclosing them in folded DNA structures. In addition, these aptasensors generally present the problem of substantial background current [15].

Based on this knowledge, Zhang and coworkers [16] reported on an electrochemical DNA biosensor based on a hairpin anti-OTA aptamer. Using this design, their DNA biosensor detected OTA in wheat samples as low as 0.4 ng/L for OTA with a linear range of 1–20 ng/L. Wu and coworkers [17] developed a one-step electrochemical aptasensor for rapid and ultrasensitive OTA detection by employing the principle of the binding-induced conformational change of the aptamer against OTA. The electrochemical aptasensor method developed in this way permitted the detection of OTA with a sensitivity of 0.095 ng/L for the LOD and 0.1–1 ng/L as the linear range. The assay was used to monitor the OTA in red wine samples. Recently, Hayat and coworkers [18] designed two strategies to construct structure-switchable electrochemical label-free aptasensors for small-size molecule detection. The fabrication design for the two aptasensors was demonstrated by the detection of OTA. In the first strategy, a long spacer chain of polyethylene glycol (PEG) was immobilized on a screen-printed carbon electrode (SPCE) via electrochemical oxidation of its terminal amino group, and subsequently the amino-aptamer was immobilized on the modified surface to form a two-piece macromolecule. The immobilized macromolecules designed in this way resulted in the formation of long tunnels on the SPCE surface, whereas the aptamer acted as the gate for the tunnels. The aptamer gates were closed due to a change in conformation of the aptamer on target analyte binding, which decreased the electrochemical signal. The decrease in the electrochemical signal was linear and in the range of 0.12–5.5 ng/L with a detection limit of 0.12 ng/L. This method was tested in beer samples [18]. The second platform was based on the direct chemistry of hexamethylene diamine via electrochemical oxidation of its terminal amino groups on SCPE. The activated carboxyl aptamer was covalently linked to another amino-terminal group of the immobilized hexamethyldiamine to design a structure-switchable aptasensor for OTA detection. The decrease in the electrochemical signal was due to aptamer–analyte binding and used to measure the OTA concentration. The aptasensor showed an LOD of 0.1 μg/L with a linear range of 0.12–8.5 μg/L, and it was validated in beer samples [19].

Here we describe a new simplified PEG aptamer (PEG-Apt), "two-piece macromolecules," which exploits a conformational change in the aptamer that takes place when this macromolecule binds to a target analyte. In this fabrication scheme, heterobifunctional linear and long spacer arms of PEG were obtained on the electrode surface via electrochemical oxidation of NH_2–PEG–COOH. The amino-aptamer was linked flexibly to the terminal carboxylic group of the PEG spacer, forming a diblock macromolecule immobilized on the BDD surface. To our knowledge, this is the first PEG aptasensor prepared on a diamond surface. The diblock macromolecules were expected to form autoassembled long-chain spacer arms on the substrate surface, forming a dense layer through which electron transfer would take place. In the absence of the target analyte, the aptamer remains unfolded and, therefore, rapid electron transfer to the electrode surface is allowed. On target analyte binding, the electron transfer is inhibited due to conformational changes to the aptamer, which transforms the random coils to the G-quadruplex structures that block the electron transfer to the electrode surface.

By employing this strategy, we have demonstrated that our designed electrochemical aptasensor is simple, sensitive, and stable. In comparison with existing aptasensors based on conformational changes, the proposed strategy offers substantial advantages because the target inducing conformational changes is used to detect the target analyte, thereby compensating for the size factor of the molecules. Owing to the excellent performance of the prepared aptasensor, a wide linear range and a much lower detection limit of OTA with good selectivity were obtained. The developed method was also used to detect OTA in field samples with sensitivity in the range of pg/ml.

Materials and methods

Chemicals and materials

Polyethylene glycol 2-aminoethyl ether acetic acid NH_2–PEG^{77}–COOH (MW = 3000), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC), potassium ferrocyanide [$K_4Fe(CN)_6$], and potassium ferricyanide [$K_3Fe(CN)_6$] were purchased from Sigma (France). OTA (from *Aspergillus ochraceus*) and ochratoxin B (OTB) were also obtained from Sigma; they were dissolved in methanol and then diluted in binding buffer. The anti-OTA modified aptamer was purchased from Eurogentec (France). The binding site of the aptamers is identical to that reported in the literature [20]. The aptamer sequences are shown below:

(5′-GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACA-3′)-5′-NH_2.

Aptamer solutions were prepared in binding buffer (pH 7.4) containing 1 mM $MgCl_2$, 140 mM NaCl, 2.7 mM KCl, 0.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4.

Instrumentation

Preparation of BDD electrochemical cell

Electrochemical microcells were made from a film of 300 nm microcrystalline BDD deposited on an insulated silicon wafer, 4 inches in diameter, provided by NEOCOAT (La Chaux-de-Fonds, Switzerland). The 300-nm-thick polycrystalline BDD with a boron concentration higher than 7000–8000 ppm was grown by MPECVD (microwave-assisted plasma-enhanced chemical vapor deposition) on silicon that was previously coated with an insulating layer of silicon oxide and silicon nitride ($Si/SiO_2/Si_3N_4$) 0.5 μm thick. The three electrodes—working electrode (1 mm in diameter), counter electrode, and pseudo-reference electrode—were cut out of the BDD wafer by micromachining [21]. This was done by the MANUTECH USD (Saint-Etienne, France) using a femtosecond laser (5 kHz, 2.5 W, 800 nm, and 150 fs), a scanner head, and a set of XYZ