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Highly sensitive ochratoxin A impedimetric aptasensor based on the immobilization of azido-aptamer onto electrografted binary film via click chemistry

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ABSTRACT

The aptamer immbolization onto organized mixed layers of diazonium salts via click chemistry was explored. The immobilized aptamer was employed in the fabrication of a highly sensitive and reusable electrochemical impedimetric aptasensor for the detection of ochratoxin A (OTA). The screen-printed carbon electrodes (SPCEs) were first modified by electrografting of a protected 4-((trimethylsilyl)ethynyl) benzene (TMSi-Eth-Ar) layer followed by a second one of *p*-nitrobenzene (*p*-NO_2-Ar) by means of electrochemical reduction of their corresponding diazonium salts, (TMSi-Eth-Ar-N_2^+) and (*p*-NO_2-ArN_2^+). After deprotection, a layer with active ethynyl groups was obtained. In the presence of copper (I) catalyst, the ethynyl groups reacted efficiently with aptamer bearing an azide function, thus forming a covalent 1,2,3-triazole linkage. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) in the presence of ferri/ferrocyanide redox probe $[Fe(CN)_6]^{4-/3-}$ were used to characterize each step in the aptasensor development. The increase in electron-transfer resistance (R_{et}) values due to the specific aptamer–OTA interaction was proportional to the concentration of OTA in a range between 1.25 ng/L and 500 ng/L, with a detection limit of 0.25 ng/L.

1. Introduction

Aptamers are nucleic acid ligands which are isolated from random-sequence DNA pools by a technique known as SELEX (systematic evolution of ligands by exponential enrichment) or *in vitro* selection [1]. Aptamers are capable of binding tightly and specifically to targets ranging from small molecules to complex multimeric structures [2,3]. Aptamers were used as affinity ligands instead of monoclonal and polyclonal antibodies, as a promising alternative for many target molecules [4,5]. Up to now, most of the developed aptasensors are based on optical and electrochemical transduction methods. Electrochemical aptasensors have been appeared as promising bio-tool due to their low limit of detection and high sensitivity. In order to increase the system sensitivity, many electrochemical aptasensors are fabricated by labeling the aptamers with electroactive materials such as enzyme, ferrocene and methylene blue [6–8]. However, the labeling of aptamer makes the assays more complex, time consuming and laborious. Moreover, labeling process affects the binding affinity between the targets and their aptamers to a certain degree [9,10]. In an effort to overcome these drawbacks, it is important to develop label free and highly sensitive electrochemical aptasensors. Among the label free methods, electrochemical impedance spectroscopy (EIS) has attained growing attention because of high sensitivity, low cost, fast response time and simple equipment. It has been proven that EIS can be successfully used as an enzyme sensor [11], immunosensor [12] and DNA sensor [13]. In the present work, a label free impedimetric aptasensor based on the immobilization of azido-aptamer onto binary film via click chemistry was explored to detect ochratoxin A (OTA). The electrochemical grafting of binary film and click chemistry employed here were expected to provide uniform, controlled and efficient immobilization of aptamer to improve the sensitivity of the system, in addition to reducing the non-specific signal.

Click chemistry is attracting lot of interest and importance in recent years [14,15]. As an azide/alkyne 1,3-dipolar cycloaddition, it was first introduced by Huishgen in 1984 as a reaction at high temperature in organic solvent [16]. In 2001, Sharpless et al. performed this reaction in aqueous phase with Cu (1) as catalyst under very mild conditions [17]. From then on, click chemistry received vital importance not only because it is irreversible, quantitative and mildly processed, but also because the 1,2,3-triazole ring formed in the reaction is similar to peptide bond in atom placement and electronic properties. The ring like peptide

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helps to maintain biological activity of the immobilized biomolecule [18,19]. Moreover, this heterogeneous coupling reaction is fast, resistant to side reactions, selective, compatible to various solvents (including water), reproducible, highly tolerant to reaction conditions and has high yield. Additionally, the formed 1,4-disubsituted 1,2,3-triazole is very stable under physiological conditions, and the azides are highly energetic and inert to biomolecules, which is useful for site-specific immobilization of biomolecules onto solid surface [20]. These advantages make click chemistry suitable for electrochemical grafting of biomolecules onto electrode surface. Although covalent attachment based on click chemistry for enzymes [21,22] and proteins [14] was performed, the concept of click chemistry for aptamer immobilization is new, and not reported in the literature.

Similarly, potential and interesting applications can be obtained only when biomolecules are properly immobilized on solid surface. Recently, the direct and covalent immbolization of biomolecules have been used to functionalize solid interface, such as carbon, silicon, metals, and diamonds [23]. However, covalent immobilization is difficult to control and yields randomly bound biomolecule with poor orientation or inappropriate alignment, which results in inefficient electron transfer to electrode surface [21]. An alternative strategy was recently developed by Leroux et al., who successfully used a binary layer containing two reagents to obtain the uniform, compact and controlled modified electrode surface [24]. On the basis of this concept, we explored here the use of two binary films of diazonium salts in the construction of aptasensor. It was expected that the controlled and uniform modified electrode surface would provide the systematic immobilization of aptamer, to improve the system reproducibility and sensitivity. It is worth to note that the concept of click chemistry and electrode surface modification with binary films is reported for the first time in the construction of aptasensor.

Ochratoxin A was selected as target molecule for this study because it contaminates a variety of food commodities. OTA has several toxicological effects such as nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic and it is believed to cause increased oxidative stress at a cellular level [25,26]. The European Union has introduced some regulatory limits to control the level of OTA in food stuff such as raw cereal grains (5 μg/kg), dried fruits (10 μg/kg), roasted coffee and coffee products (5 μg/kg), grape juice (2 μg/kg) (EC No. 123/2005) and also for all types of wines (2 μg/kg).

2. Experimental

2.1. Materials and reagents

The azido and amino modified aptamers were purchased from Eurogentec (France). The binding site of the aptamers is identical with that reported in [31]. The aptamer sequences are show below

5′-GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACA-3′), 5′-N_3
5′-GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACA-3′), 5′-NH_2

All other chemicals, potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide ($K_4[Fe(CN)_6]$), sodium phosphate dibasic Na_2HPO_4, potassium phosphate monobasic KH_2PO_4, sulfuric acid (98%), ethanol (98%), and 4-((trimethylsilyl) ethynyl) aniline (TMSi-Eth-Ar-NH_2), 4-nitroaniline (*p*-NO_2-Ar-NH_2), sodium nitrite, ochratoxin A (OTA) (from *Aspergillus ochraceous*) and ochratoxin B (OTB) first dissolved in methanol and then diluted in binding buffer, Horseradish peroxidase (HRP, EC 1.11.1.7), tris(hydroxymethyl) aminomethane $(HOCH_2)_3$ CNH_2, Disodium ethylenediaminetetraacetate dihydrate EDTA disodium salt, EDTA-Na_2, (+)-sodium L-ascorbate, copper(II) sulfate pentahydrate and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, were all supplied by Sigma-Aldrich. Aptamer solutions prepared in binding buffer (BB) 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 were used. The azido horseradish peroxidase (N_3-HRP) was synthesized according to the published procedure [27]. All solutions were prepared with deionized Milli-Q water (Millipore, Bedford, MA, USA).

2.2. Apparatus

The electrochemical measurements were carried out with an Autolab PGSTAT100 potentiostat/galvanostat (Eco Chemie, The Netherlands) with computerized control by GPES 4.9 and FRA software, connected to screen printed carbon electrode (SPCE), which comprises a working carbon electrode, printed from a carbon-based ink, a pseudo reference electrode made from a silver-based ink, and the auxiliary electrode from a carbon ink. The impedance spectra were recorded using a sinusoidal ac potential perturbation of 5 mV (rms), in the frequency range 10^4–0.5 Hz, superimposed on a dc potential of 0.095 V, and readings were taken at 20 discrete frequencies per decade. All measurements were performed in a solution of 1.0 mM ferri/ferrocyanide couple $[Fe(CN)6]^{4-/3-}$ in PBS, pH 7.3, as a background electrolyte.

2.3. Functionalization of SPCE surface with binary film of diazonium salts

The TMSi-Eth-Ar-N_2^+ ions were synthesized *in situ* by reaction of 2.0 mM TMSi-Eth-Ar-NH_2 and 2.0 mM sodium nitrate in 0.5 M HCl solution for 5 min. The electrochemical modification of SCPE with *in situ* generated TMSi-Eth-ArN_2^+ ions was performed by one potential cycling between 0.4 and −0.5 V. The modified SCPEs were subsequently treated with ethanol solution for 10 s to wash any physically adsorbed species. The *p*-NO_2-ArN_2^+ ions, obtained by the *in situ* reaction of 4-nitroaniline (2.0 mM) and sodium nitrate (2.0 mM) in 0.5 M HCl solution, were electrochemically immobilized onto TMSi-Eth-Ar-SPCE electrode. This protected surface TMSi-Eth-Ar-pNO_2-Ar-SPCE was treated with tetrabutylammonium fluoride (TBAF) to remove the TMSi group, and to obtain an ethynyl-modified surface.

2.4. Covalent immobilization of aptamer via click reaction

The immobilization of aptamer was performed by immersing the above ethynyl-modified SPCEs in azido-aptamer solution (0.5 μM) with sodium ascorbate (10.0 mM) and copper (II) sulfate pentahydrate (1.0 mM). The electrode with azido-aptamer solution was treated at −200 mV *vs.* pseudo ref. silver electrode for 5 min. The EDTA solution was used to remove the physically absorbed azide moieties and excess copper. For the electrochemical experiments, a 100 μL of OTA dissolved in BB was dropped onto the surface of the sensor, followed by incubation for 60 min in different concentrations of OTA (0.0–500 ng/L).

2.5. Regeneration of the impedimetric aptasensor

The electrodes were rinsed with distilled water after OTA incubation, and immediately used for electrochemical experiments. After each use, the electrodes were regenerated by washing with 100 μL of regeneration solution for 30 s The regenerating buffer was prepared according to a previously described protocol for regenerating the solid phase extraction column (methanol: eluting buffer (10.0 mM TRIS, 1.0 mM EDTA, pH 9.0) (20:80,v/v) [28].