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Sensitive analytical performance of folding based biosensor using methylene blue tagged aptamers

Gaëlle Catanante [a,1], Rupesh K. Mishra [a,*,1,2], Akhtar Hayat [a,b], Jean-Louis Marty [a,*]

[a] Laboratoire B.A.E., Université De Perpignan Via Domitia, 52 Avenue Paul Alduy, Perpignan Cedex 66860, France
[b] Interdisciplinary Research Centre in Biomedical Materials (IRCBM), COMSATS Institute of Information Technology (CIIT), Lahore 54000, Pakistan

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ABSTRACT

This work demonstrates the development of a folding based electrochemical aptasensor using methylene blue (MB) tagged anti-Ochratoxin A (OTA) aptamers. Different aptamer coupling strategies were tested using Hexamethylenediamine, polyethylene glycol, simple adsorption and diazonium coupling mechanism. The best sensitivity was recorded by oxidation of amines using hexamethylenediamine (HDMA) on screen printed carbon electrode (SPCE). To achieve the direct detection of OTA, aptamer conjugated redox probe was used and detection was demonstrated based on the conformational changes in aptamer structure upon OTA sensing. Signaling in this class of sensors arises from changes in electron transfer efficiency upon target-induced changes in the conformation/flexibility of the aptamer probe. These changes can be readily recorded electrochemically. The developed aptasensor is unique in its own mechanism as redox probe tagged aptamer coupling such as MB has never been tried to immobilize using long carbon chain spacers as, addition of spacers would provide more sensitive detection methods. A good dynamic range 0.01–5 ng/ml was obtained for OTA with Limit of detection (LOD) 0.01 ng/ml and Limit of quantification (LOQ) of 0.03 ng/ml respectively. The good reproducibility was recorded with RSD% of 3.75. The obtained straight line equation was $y=0.4035x+0.90311$, $r=0.9976$. We believe that the sensor design guidelines outlined here represents a general strategy for developing new folding-based electrochemical aptasensors. The developed aptasensor was extended to screen cocoa samples for OTA contamination. The cocoa samples were extracted and purified using molecular imprinted polymer (MIP) columns. The aptasensor displayed good recovery values in the range 84–85% thus, exhibited the effectiveness of proposed aptasensor for such complex matrices.

1. Introduction

Electrochemical, aptamer-based (EAB) biosensors employing structure-switching aptamers represent a propitious stage for the rapid and sensitive quantification of target molecules [1]. To date, aptamer based biosensors have been reported against a wide range of targets including bacterial, proteins [2–5], ions, small molecules [6], and mammalian cells [7,8]. This is achieved through the utilization of single-stranded DNA or RNA aptamers that have been selected for binding to a specific target [8]. The principal signaling mechanism in this class of sensors is a result of conformation and/or flexibility changes in the electrode bound aptamer [4,9]. Typically, the aptamer is modified at the 5′-end for electrode attachment (e.g., thiolated) and at the distal, 3′-end, with a redox probe molecule such as methylene blue (MB) or ferrocene [10–14]. Because the electron transfer efficiency is a function of the distance and collision frequency between the redox reporter and the electrode surface, changes in faradaic current upon target binding is readily measured electrochemically [15,16]. Target detection is performed by measuring changes in voltammetric peak current, typically defined as percent signal change [17,18]. Using this sensing strategy, EAB sensors have been reported to exhibit typical limits of detection (LOD) from micromolar down to picomolar levels when detecting, for example, proteins [19] or small molecules [11]. In addition, sensors are able to perform such detection in complex sample matrices, whole blood or physiological matrix [5,20,21].

Although such kind of electro-chemical aptasensors has been fabricated for selective and sensitive detection of many target molecules, they required a large scale conformational change of the aptamer to modulate the distance of redox tags from the electrode to change the signal [20]. Additionally, these aptasensors generally undergo the problem of substantial background current [22]. To circumvent these drawbacks, herein, we described a new simplified approach using spacer attached aptasensors. In this

* Corresponding authors.
[1] Equal contributors to the work.
[2] Department of Biosciences and Biotechnology, Banasthali University, Rajasthan, India 304022 (present address).

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fabrication scheme, hetero bifunctional linear and long spacer arm of different molecular weight Poly ethylene glycol (PEG) and hexamethyldiamine (HMDA) was obtained on electrode surface via electrochemical oxidation of NH_2–PEG–COOH. The amino-aptamer was linked flexibly to terminal carboxylic group of PEG spacer forming a diblock macromolecule that is immobilized on screen printed carbon electrode (SPCE) surface. The diblock macromolecules were expected to form clusters of long chain spacer arms on the substrate surface, leaving long hole for the redox probe to reach the electrode surface. In the absence of target analyte, the aptamer remained unfold, thereby allowing the passage of redox probe to electrode surface [23]. Upon target analyte binding, the electron transfer is inhibited due to binding conformational changes of aptamer from random coils to G-quadruplex structure that significantly covers the entrance of long tunnel, blocking redox probe to reach the electrode surface [24].

It is difficult to achieve ultrasensitive detection of small molecules by a basic aptasensor because of the low size of analytes. Thus, in order to carry out the sensitive detection, the exploration of novel methods is essential. Here, we employed our designed platform to develop a simple and ultrasensitive electrochemical aptasensor for the small size target analyte. Ochratoxin A (OTA) (408 MW), it contaminates a variety of food commodities [25]. Cocoa and various derivatives contamination (International Cocoa Organization (ICCO) with OTA has been revealed by numerous studies using conventional techniques [26–28]. OTA has several toxicological effects such as nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic, and it is believed to cause increased oxidative stress at cellular level [29,30]. Recently, numerous aptamer based biosensors based on enzyme-labels, nano particles and label free detection methods have been exploited for OTA detection [31–34]. By employing this strategy, we demonstrated that our designed electrochemical aptasensor is simple, sensitive and stable. Compared to existing aptasensors based on conformational changes, the proposed strategy offers substantial advantages because the target induced conformational changes is used to cover the entrance of a long tunnel to detect the target analyte, eliminating the size factor of molecules.

In this work, we have demonstrated the OTA detection in cocoa samples. Cocoa beans after post-harvest treatments are mainly exported to Europe and North America to be turned into liquor, butter and cocoa powder [35]. Cocoa and various derivatives contamination (International Cocoa Organization [ICCO] with OTA has been revealed by numerous studies using conventional techniques. Hence, there is a need to screen cocoa beans for OTA quantitation before consumption. Cocoa is a complex matrix with tannin and a thick surface resulting in turbid samples even after the extraction using suitable chemicals. Herein, we have used 1% sodium hydrogen carbonate ($NaHCO_3$) for extraction and Molecular imprinted polymer (MIP) columns were employed for sample clean-up. MIP columns are cost effective, chemically stable and compatible with all solvents [36]. The developed method combines the significant advantages of differential pulse voltammetry (DPV) with a rapid and real-time monitoring of OTA using aptamers. DPV offers high sensitivity, low limit of determination, easy operation, and the use of simple instrumentation [34,37]. The exploration of MIP columns, SPEs and the optimization of appropriate aptamer concentrations will certainly make an impact on real tests.

2. Chemicals and materials

Hexamethylenediamine, N-Hydroxysuccinimide (NHS), N-(3-dimethylaminopropyle)-N′-ethyle-carbodiimide hydrochloride (EDC), were purchased from sigma (France). Ochratoxin A (from *A. ochraceous*) and Ochratoxin B (OTB) procured from Sigma was first dissolved in methanol and then diluted in binding buffer. The anti-OTA aptamer tagged with redox probe methylene blue was ordered from Microsynth, Switzerland. The aptamer sequences are shown below as 5' end is modified with $COOH^-$ and 3′ was methylene blue tagged.

5′-GAT CGG GTC TGG GTG GCG TAA AGG GAG CAT CGG ACA-3′ ($COOH^-$OTA-MB).

Aptamer solutions were prepared in binding buffer (BB), pH7.4 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. NH_2-PEG-COOH (300) and PEG 24-Amine (MW 1146.25), 4-amino benzoic acid, sodium nitrite ($NaNO_2$), hydrochloric acid ethanolamine and bovine serum albumin were purchased from Sigma (France).

2.1. Apparatus

All electrochemical measurements were carried out using an AUTOLAB PGSTAT100 potentiostat/galvanostat equipped with a frequency response analyzer system (Eco Chimie, Netherlands) controlled by General purpose Electrochemical system (4.9) for voltammetry. SPCEs were fabricated using a DEK 248 screen-printing system [38]. The SPCE consists of conventional three electrode configuration with graphite as working (4 mm diameter disk) and counter (16 mm × 1.5 mm curved line) electrode, and Ag/AgCl (16 mm × 1.5 mm straight line) as pseudo-reference electrode.

2.2. Electrochemical oxidation of amines on a SPCE surface

Electrochemical grafting of long spacer chains to SPCE was carried out in 0.05 M of Tetrabutylammonium tetra-fluoroborate 99% ($NBu4BF_4$) − 0.5 M acetonitrile solution containing 6 mM hexamethyldiamine (HMDA), 6 mM NH_2-PEG-COOH (MW3400) or carboxy PEG amine (10,000). Then the potential was cycled between 0.3 V and 1.7 V (vs. SPCE) at a scan rate of 0.5 V/s for the electrochemical oxidation of amino groups. After washing the electrode surface with water, the modified electrodes were used directly for aptamer immobilization or stored at room temperature without loss of any immobilization efficiency. After rinsing the electrode with water to remove the unbound aptamer, the anti-OTA-aptamer modified SPCEs can be used directly for aptasensing or stored dry at 4 °C for several days without any decrease in the sensitivity. For the electrochemical experiments, a 100 mL of OTA dissolved in BB was dropped onto the surface of the sensor, followed by incubation for 45 min in different concentrations of OTA.

2.3. Covalent immobilization of MB tagged aptamer

The redox probe MB was tagged on 3′ of the aptamer whereas 5′ was modified with $COOH^-$. Prior to the immobilization, the terminal carboxylic group of aptamer at 5′ was activated by 100 mM EDC and 25 mM NHS in 100 mM MES buffer for 60 min. After the activation process, 20 mL of activated aptamer solution (2 μM) were incubated onto the SPCE surface for 45 min under water saturated atmosphere. After rinsing the electrode with water to remove the unbound aptamer, the anti-OTA-aptamer modified SPCEs can be used directly for aptasensing, or stored dry at 4 °C for several days without any decrease in the sensitivity. For the electrochemical experiments, a 100 mL of OTA dissolved in BB was dropped onto the surface of the sensor, followed by incubation for 45 min with different concentrations of OTA.

2.4. Diazonium coupling and adsorption

In another approach, the clean SPCE surface modification was