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An electrochemical aptasensor based on graphene doped chitosan nanocomposites for determination of Ochratoxin A

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ABSTRACT

A simple and efficient functionalized graphene (f-graphene) doped chitosan (CS) based electrochemical aptasensor has been developed for ochratoxin A (OTA) detection. Use of f-graphene increased the electroactive surface area of the electrode and CS prevented leaching of the aptamer (APT) molecules. The dual properties of CS and f-graphene overall improved the sensor performance. The developed APT/SA/CS-f-graphene sensor has been characterized using Fourier Transform Infrared Spectroscopy (FTIR), Contact angle, Field emission - scanning electron microscopy (FE-SEM) and electrochemical studies. The aptasensor displayed OTA limit of detection (LOD) about 1 fg/mL for standard and 0.01 ng/mL for spiked sample within response time of 8 min. The reusable aptaelectrode retained 85% of initial current values after 7th reaction with stability of about 7 days. Besides, real application of the fabricated aptasensor has been evaluated in the grape juice samples. Recoveries of OTA in the range of 90–101% were estimated successfully.

1. Introduction

Ochratoxin A (OTA), (2*S*)-2-[[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydroisochromene-7-carbonyl]amino]-phenylpropanoic acid, is a toxic secondary metabolite produced mainly by filamentous fungus species like *Aspergillus ochraceus*, *Aspergillus niger* and *Penicillium verrucosum* [1]. The mycotoxin OTA is a cyclic pentaketide, dihydroisocoumarin derivative linked to phenylalanine moiety. Excessive usage of OTA causes lethal effects such as teratogenic, nephrotoxic, hepatotoxic, neurotoxic, genotoxic and immunosuppressive effects [2]. According to European Commission, the valuated maximal admissible levels of OTA for different food stuffs were: 3 μg/kg for cereal products, 5 μg/kg for unprocessed cereal products (rice and buckwheat) and 10 μg/kg for dry grapes [3].

For the prevention against the severe toxic effects of OTA, a highly sensitive and rapid sensing tool to monitor OTA in food products is required. Although there are several techniques such as HPLC-fluorescence detector (FAD) [4], LC-MS/MS [5], TLC-charged couple detector (CCD) [6] and GC–MS [7] to detect the OTA contamination. But, these methods are expensive, laborious, time-consuming and require qualified technical staff.

In the recent years, biosensors have emerged as a powerful and efficient tool for the detection of mycotoxins. There are already many reports available related to the detection of OTA based on competitive inhibition immunoassay using SPR [8], antibody conjugated electrochemical biosensors [9] and fluorescence-linked immunosorbent assay [10]. Although immunoanalytical techniques are highly sensitive, they do have limitations in terms of cross-reactivity, matrix interference, false screening results, require high quality antibodies whose preparation via immunization is time-consuming and susceptible to instability. To overcome these, aptamer (APT) based electrochemical biosensors are the current area of research nowadays in the field of clinical diagnosis, food-industry and environmental monitoring as these APTs are cost-effective, stable in terms of temperature, pH and specific activity as compared to antibodies [11]. A number of nanomaterials such as gold, silver, zinc oxide, iron oxide, graphene and its derivatives have been explored in the fabrication of aptamer based biosensor. Graphene oxide is used for fabrication of various electrochemical sensors due to its ability for promoting electron transfer [12–14]. Besides carbon nanomaterials, Chitosan (CS) has also been brought into sharp focus as a suitable matrix due to its biocompatibility, hydrophilicity, non-toxicity, excellent mechanical stability, cost-effectiveness and availability of reactive functional groups for chemical modifications. Immobilization of the biomolecules on a suitable matrix plays a crucial role in the performance of a biosensor. The dual use of CS and f-graphene

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facilitates the more active sites for the immobilization of APT molecules [15]. The performance potential of the CS and graphene has been explored by Tabasi et al. for the fabrication of an aptasensor for detection of human epidermal growth factor receptor 2 protein (HER2) cancer biomarker. The obtained LOD was 0.21 ng mL^{-1} with sensitivity of 0.14 μA ng^{-1}mL [16]. Further, Thakur et al. investigated the role of Graphene and iron oxide doped chitosan as immobilization matrix in the development of an aptasensor for determination of *M. tuberculosis* antigen MPT64. The potential of chitosan and graphene is evident from the LOD (0.5 fg/mL) and response time of 15 min [17].

Studies are available on the APT based electrochemical biosensors for detection of OTA. An ultrasensitive aptasensor for OTA using hexagonal core/shell up conversion nanoparticles tagged OTA APT as electron donor and graphene oxide as electron acceptor has been developed [18]. Catanante et al. have developed a folding based electrochemical aptasensor using methylene blue tagged anti-OTA aptamers. It showed an analytical limit of detection as 0.01 ng/mL [19]. Sun et al. designed a thionine-aptamer/graphene nanocomposite for OTA detection at a concentration as low as 5.6 pg/mL [20]. An immobilization-free homogeneous indium tin oxide (ITO) based electrochemical aptasensor with high efficiency of exonuclease-catalyzed target recycling and high selectivity of the aptamer against OTA has been reported with limit of detection as 0.004 ng/mL [21]. APT immobilized graphene quantum dots doped silica nanoparticles have been used to sense OTA in combination with electro-chemiluminescence and fluorescence [22]. Several other biosensors including impedimetric immunosensor [23–26], piezoelectric immunosensor [27] and enzyme-based biosensor [28] have also been emerged for OTA detection. However, the electrochemical aptasensors offer new opportunities to OTA detection due to their simplicity, high specificity, sensitivity, easy use, low cost, possible miniaturization, compatibility with fabrication techniques and portability, which are basal requirements for point-of-care (POC) applications. In the present study, electrochemical aptasensor based on f-graphene doped CS has been developed for sensitive detection and determination of OTA.

2. Experimental

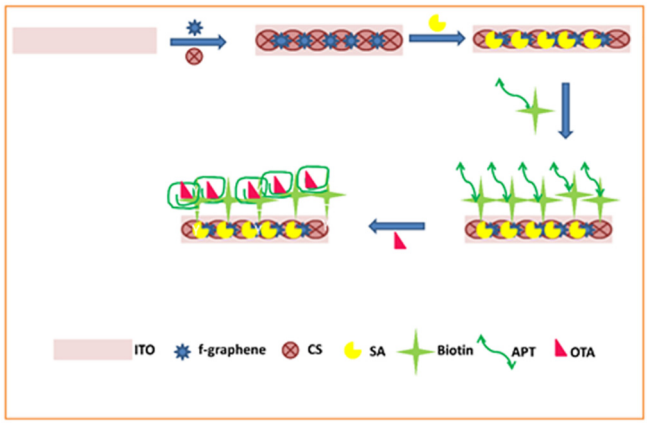
2.1. Reagents

Graphite powder, biotinylated DNA APT sequence (36 mer), OTA, Streptavidin (SA), N-[3-(dimethylaminopropyl)-N-3-ethylcarbodiimide hydrochloride] (EDC), N-hydroxysuccinimide (NHS) and indium doped tin oxide (ITO) sheets were purchased from Sigma-Aldrich (USA). Chitosan (CS) was purchased from HiMedia Laboratories Pvt. Ltd. USA. Autoclaved buffer solutions were used in all experiments. Phosphate Buffer Solution (PBS) has been prepared by mixing the stock solutions of 0.1 M monosodium phosphate (NaH_2PO_4) and disodium phosphate (Na_2HPO_4). All other chemicals used were of analytical grade. The deionized water has been used throughout the experiments. APT sequence specific to OTA was taken from the previously reported literature [29].

Biotinylated APT sequence-5′-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3′.

2.2. Preparation of graphene and its chemical functionalization

Graphene was prepared by chemical vapour deposition method as reported in literature [30]. 2 g of graphene mixed in H_2SO_4 and HNO_3 (3:1) was refluxed at 40 °C for 16 h. Repeated washing was done by centrifugation at 8000 rpm (5641 rcf) for 15 min with water until pH reduced to 7.0. The prepared f-graphene was dried at 60 °C. For the group activation, 2 mg of f-graphene was sonicated for 30 min and treated with 5 mg/mL of NHS and EDC solution for 4 h. The treatment with NHS and EDC activated the COOH groups of graphene for further binding with the NH_2 groups of SA. UV characterization was done to affirm the successful formation of f-graphene (Fig. S1) using UV spectrophotomete (Ultrospec 7000).



Scheme 1. Stepwise performance and illustration of the fabrication of an aptasensor towards OTA detection.

2.3. Fabrication of aptasensor and characterization studies

CS solution was prepared by dissolving 2 g of CS in 1% acetic acid and ultrasonicated at room temperature for 1 h till the solution became transparent. The solution was left undisturbed to settle the impurities if present. The CS-f-graphene suspension was prepared by dispersing 1 mg/mL of f-graphene in CS solution and sonicated for 2 h. The electrodes were prepared by dip coating the ITO sheets in the above prepared solution. The electrodes were further modified with 8 μL of SA solution (0.05 mg/mL). These SA immobilized CS-f-graphene electrodes were kept in humid chamber at 4 °C for overnight and rinsed with deionized water to remove any unbound SA molecules. The biotinylated single stranded (ss) DNA APT was immobilized on the surface of modified electrode (SA/CS-f-graphene) via biotin-SA interaction for 12 h at 4 °C. The APT immobilized electrode was washed twice with distilled water before use to remove any unbound APT molecule. Scheme 1 illustrates the stepwise performance of an aptasensor towards OTA detection.

The electrodes were characterized by Fourier Transform Infrared Spectroscopy (FTIR) (Nicolet 1S50 FTIR), Field emission - scanning electron microscopy (FE-SEM, Hitachi SU 8010), Contact angle (Sessile drop method [KRUSS Drop shape analysis system] Model DSA100) (Fig. S2) and electrochemically (Potentiostat/Galvanostat, AutoLab Eco-Chemie, Netherlands) to affirm the successful fabrication of aptasensor.

3. Results and discussion

3.1. Characterization studies

3.1.1. FTIR studies

The FTIR spectra of bare CS/ITO (Fig. 1, curve a) displayed the broad band between 3200 and 3400 cm^{-1} due to stretching vibrations mode of OH groups [31]. The bands at 1690 and 1480 cm^{-1} is due to the vibrations of protonated amine group. Further, due to the bending vibrations of methylene and methyl groups, the bands appeared at 1375 and 1426 cm^{-1} and at 1150 cm^{-1} is related to the asymmetric vibrations of CO in the oxygen bridge resulting from deacetylation of CS [32]. The peak at 890 cm^{-1} is correlated to the wagging of the saccharide structure of CS [33]. The f-graphene displayed peak at 1680 cm^{-1} because of −C═O stretch in −COOH acids (curve b). In FTIR spectrum of CS-f-graphene/ITO bioelectrode, the IR bands subjected to carboxylation at 1700 cm^{-1} and in between 2800 and 3500 cm^{-1} because of C═O stretch in carboxylic acids (curve c) [34]. These −COOH bands got slightly shifted to newer position in