



# One pot synthesized zirconia nanoparticles embedded in amino functionalized amorphous carbon for electrochemical immunosensor



Pramod K. Gupta<sup>a,b</sup>, Namrata Pachauri<sup>a</sup>, Zishan H. Khan<sup>b,\*</sup>, Pratima R. Solanki<sup>a,\*</sup>

<sup>a</sup> Special Centre for Nanoscience, Jawaharlal Nehru University, New Delhi 110067, India

<sup>b</sup> Department of Applied Sciences and Humanities, Jamia Millia Islamia, New Delhi 110067, India

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## ABSTRACT

Zirconia nanoparticles embedded in amino functionalized amorphous carbon (nZrO<sub>2</sub>-NH<sub>2</sub>C) nanocomposite were synthesized by a novel, simple and one-pot hydrothermal method. The hydrothermal synthesis process involved carbonization of chitosan into amino functionalized amorphous carbon (NH<sub>2</sub>C) and simultaneously *in-situ* growth of zirconia nanoparticles (nZrO<sub>2</sub>). The existence of carbon into the nanocomposite during the synthesis enhanced mechanical strength of nZrO<sub>2</sub>. Beside this, the presence of NH<sub>2</sub>C in nanocomposite minimized the aggregation of nZrO<sub>2</sub> (~10 nm), and hybrid interface of nZrO<sub>2</sub>-NH<sub>2</sub>C that enhanced the electrochemical properties. The NH<sub>2</sub>C also improved the wettability of the nanocomposite as observed by contact angle measurement. This nZrO<sub>2</sub>-NH<sub>2</sub>C nanocomposite was deposited onto indium tin oxide (ITO) coated glass substrate using the electrophoretic technique. The oxygen moieties of nZrO<sub>2</sub> and amine groups of NH<sub>2</sub>C in nanocomposite formed an admirable microenvironment for immobilization of anti-OTA (antibodies specific to Ochratoxin A). XPS and FTIR studies revealed the presence of amine groups on nZrO<sub>2</sub>-NH<sub>2</sub>C nanocomposite and immobilization of biomolecules (anti-OTA and BSA) on its surface, respectively. The fabricated BSA/anti-OTA/nZrO<sub>2</sub>-NH<sub>2</sub>C/ITO immunosensor revealed a selective detection of OTA in the detection range from 1 to 10 ng mL<sup>-1</sup> with a sensitivity of 0.86 μA ng<sup>-1</sup> mL cm<sup>2</sup> and lower detection limit of 1 ng mL<sup>-1</sup>.

## 1. Introduction

In past decades, numerous kinds of metal oxide nanoparticles (MONPs) have been utilized for fabricating an efficient electrochemical biosensors [1,2]. Among MONPs, the bioceramic “Zirconium dioxide nanoparticles (nZrO<sub>2</sub>)” exhibited unique properties like high corrosion resistance, ionic conductivity, chemical inertness, excellent thermal stability, pH stability, lack of toxicity, higher isoelectric point, mechanical strength, low thermal conductivity and good biocompatibility [3–5]. The excellent biocompatibility of nZrO<sub>2</sub> with lack of any reported cases of toxicity, pH stability, and good electrochemical behavior makes it more reliable biomaterial for the fabrication of electrochemical biosensors [6,7]. The high isoelectric point of nZrO<sub>2</sub> attracts the biomolecules having lower values of isoelectric points. Moreover, the oxygen moieties attached with nZrO<sub>2</sub> provides an appropriate microenvironment for the binding of biomolecules (proteins, enzymes, and DNA) [8]. However, the difficulty with aggregation of nZrO<sub>2</sub> inhibits its utilization in biosensing applications [9]. The problem of aggregation can perhaps be addressed by synthesizing nZrO<sub>2</sub> based nanocomposite with a conductive substrate material. It can be

inferred that use of more conductive substrate material will also improve the electrochemical performance of the nanocomposite [10,11]. In past years, carbon-based nanocomposites have gained enormous consideration due to their promising uses in the field of electrochemical sensors [12]. The nanocomposites based on bioceramic (nZrO<sub>2</sub>) and carbon nanostructures have been approached to achieve two main objectives: improvement in mechanical properties of materials by the process of reinforcing with carbon nanostructures and functionalization of materials with improved conductivity [13,14]. The unique properties of carbon such as excellent biocompatibility, low toxicity, environmental-friendly, good chemical stability, high surface-to-volume ratio, robust mechanical strength and high electrical conductivity can be considered as preferred material for the reinforcement as well as functionalization of nZrO<sub>2</sub> in the nanocomposite [15–17]. Moreover, the hybrid interface of nZrO<sub>2</sub>-NH<sub>2</sub>C within the nanocomposite provides a higher electron transfer rate compared to individual materials [18].

Keeping this in view, herein we proposed a facile one step hydrothermal method for *in-situ* synthesis of nZrO<sub>2</sub> embedded in amino-functionalized carbon (nZrO<sub>2</sub>-NH<sub>2</sub>C) nanocomposite. During this

\* Corresponding authors.

E-mail addresses: [zishanhk@jmi.ac.in](mailto:zishanhk@jmi.ac.in) (Z.H. Khan), [partima@mail.jnu.ac.in](mailto:partima@mail.jnu.ac.in), [pratimarsolanki@gmail.com](mailto:pratimarsolanki@gmail.com) (P.R. Solanki).

synthesis process,  $n\text{ZrO}_2$  was formed more towards the tetragonal phase with uniform dispersion that enhanced the electrochemical properties. It is also well reported that the solubility of chitosan in acidic medium takes quite long to dissolve uniformly. The beauty of the synthesis of the  $n\text{ZrO}_2\text{-NH}_2\text{C}$  nanocomposite is no any additional requirement of acidic medium and reduced time duration to dissolve the chitosan. Besides this, a cost-effective amino functionalized  $\text{NH}_2\text{C}$  provides a significant advantage to the composite by facilitating a microenvironment to immobilize biomolecules. This  $n\text{ZrO}_2\text{-NH}_2\text{C}$  nanocomposite was electrophoretically deposited on indium tin oxide (ITO) coated glass substrate to fabricate a novel structured BSA/anti-OTA/ $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$  immunosensor for the specific detection of Ochratoxin A (OTA) using the electrochemical technique.

Ochratoxins are the secondary metabolites produced by several fungi of the *Aspergillus* or *Penicillium* families [19,20]. OTA is found in a variety of food products like coffee, beer, cocoa, pulses, dried fruits, grape juice, wine, nuts, spices and cereal products [21]. Its higher concentration in food is the most copious, toxic and it potentially affects the kidney [22]. In previous years, different nanomaterials were used for the development of an efficient electrochemical immunosensor for the OTA detection [Table S3, Supplementary data] [23–28]. Khan et al. [23,24] fabricated chitosan-polyaniline hybrid polymer (CH-PANI) and  $\text{TiO}_2$ -Chitosan based bioelectrodes in the presence of acidic medium and detected OTA using electrochemical impedance spectroscopy (EIS) technique. The use of such acidic solution can cause degradation of biomolecules. Mishra et al. [25] fabricated aptasensor to detect OTA in cocoa beans using EIS technique and found a lower detection range of  $0.15\text{--}2.5\text{ ng mL}^{-1}$ . Despite these, Jodra et al. [26] fabricated a SPCE based disposable electrochemical immunosensor for detection of OTA in coffee samples in the range of  $0.05\text{--}1\text{ }\mu\text{M}$  with higher LOD of  $1.7\text{ }\mu\text{g L}^{-1}$ . Bonel et al. [27] proposed OTA-BSA bound gold NPs modified SPCE based disposable electrochemical immunosensor for the determination of OTA in ranges from  $0.3\text{--}8.5\text{ ng mL}^{-1}$ . Recently, Mishra et al. [28] reported aptasensor for OTA detection in cocoa beans using DPV technique with a lower detection range of  $0.15\text{--}5\text{ ng mL}^{-1}$ . These above reported immunosensors for OTA detection were based on complex route of nanomaterials synthesis. In present work, *in situ* synthesized  $n\text{ZrO}_2\text{-NH}_2\text{C}$  nanocomposite was proposed for specific detection of OTA using simple differential pulse voltammetry (DPV) technique. This immunosensor showed an improved biosensing characteristic such as linear detection in the range from  $1\text{ to }10\text{ ng mL}^{-1}$ , the sensitivity of  $0.86\text{ }\mu\text{A}/\text{ng mL cm}^2$  and LOD of  $1\text{ ng mL}^{-1}$ . To the best of our knowledge, this is the first report on  $n\text{ZrO}_2\text{-NH}_2\text{C}$  nanocomposite based immunosensor for OTA detection.

## 2. Materials and methods

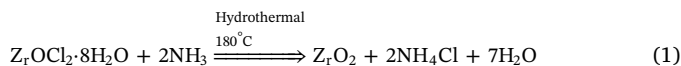
### 2.1. Materials

Zirconium oxychloride ( $\text{ZrOCl}_2\cdot 8\text{H}_2\text{O}$ ) was obtained from SRL Pvt. Ltd. India. Indium tin oxide (ITO) coated glass sheet procured from Balzers, UK. Chitosan, *N*-hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDC), bovine serum albumin (BSA), and OTA (antigen), were obtained from Sigma-Aldrich, USA. Antibody specific to ochratoxin A (anti-OTA) was procured from Abcam, USA. Ammonia solution ( $\text{NH}_3$ , 30%), ethanol ( $\text{C}_2\text{H}_6\text{O}$ ), potassium chloride (KCL), potassium ferrocyanide trihydrate [ $\text{K}_4\text{Fe}(\text{CN})_6\cdot 3\text{H}_2\text{O}$ ], and potassium ferricyanide [ $\text{K}_3[\text{Fe}(\text{CN})_6]$ ] were purchased from Fisher Scientific Ltd., India. All the chemicals were used without any further purification.

### 2.2. Synthesis of $n\text{ZrO}_2\text{-NH}_2\text{C}$ nanocomposite

The  $n\text{ZrO}_2\text{-NH}_2\text{C}$  nanocomposite was synthesized using one-step hydrothermal process. During the synthesis,  $0.1\text{ M ZrOCl}_2\cdot\text{H}_2\text{O}$  was dissolved in deionized water (40 mL) by stirring at 300 RPM for 1 h. At

this point pH of the solution was measured as 1. Then 150 mg of chitosan was added to the above aqueous solution and stirred continuously for 12 h, because of the lower pH of the  $\text{ZrOCl}_2$  solution, chitosan dissolved completely. The  $\text{ZrOCl}_2$  - chitosan aqueous solution was precipitated by dropwise addition of  $\text{NH}_3$  (30%) and solution pH was raised 10.5. The whole volume of the solution was transported into a sealed stainless steel Teflon vessel, capacity of 80 mL, and placed in a muffle furnace for 18 h at  $180\text{ }^\circ\text{C}$ . Here,  $\text{ZrOCl}_2\cdot 8\text{H}_2\text{O}$  and chitosan were used as source materials for  $n\text{ZrO}_2$  and  $\text{NH}_2\text{C}$ , respectively. Under the mild temperature ( $180\text{ }^\circ\text{C}$ ), carbonization of chitosan into the  $\text{NH}_2\text{C}$  and *in-situ* synthesis of  $n\text{ZrO}_2$  nanoparticles happened simultaneously [Eqs. (1) & (2)]. The obtained brown colored product was washed numerous times with water: ethanol to neutralize the solution pH and left for drying for 12 h at  $60\text{ }^\circ\text{C}$ .



### 2.3. Electrophoretic deposition of $n\text{ZrO}_2\text{-NH}_2\text{C}$ nanocomposite

The two electrode system was used for electrophoretic deposition (EPD) of  $n\text{ZrO}_2\text{-NH}_2\text{C}$  nanocomposite onto hydrolyzed ITO coated glass substrate. Both electrodes, ITO coated glass substrate (as a cathode), and platinum foil (as an anode) were placed parallel 1 cm to each other in a glass cell of capacity 25 mL. Before EPD, a colloidal stock solution of  $n\text{ZrO}_2\text{-NH}_2\text{C}$  ( $0.2\text{ mg mL}^{-1}$ ) acetonitrile was prepared by ultrasonication it for 2 h. During the each EPD, 4 mL of prepared colloidal solution was used under 35 V DC potential maintained between anode and cathode terminals for 3 min. For uniform and stable surface, fabricated  $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$  electrodes were washed with deionized water and dried overnight at room temperature ( $25\text{ }^\circ\text{C}$ ) in vacuum.

### 2.4. Modification of $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$ electrode with anti-OTA and BSA

A fresh stock solution of anti-OTA ( $0.1\text{ mg mL}^{-1}$ ) was prepared in phosphate buffer saline (PBS) of pH 7. The anti-OTA solution was mixed with  $0.4\text{ M EDC}$  and  $0.1\text{ M NHS}$  in the ratio of 2:1:1 and kept at  $4\text{ }^\circ\text{C}$  for 30 min, to activate the carboxyl groups in fragment crystallizable (Fc) region of anti-OTA. Immobilization of anti-OTA on the surface of  $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$  electrode was accomplished in two steps. In a first step,  $10\text{ }\mu\text{L}$  of anti-OTA with EDC-NHS was spread over the  $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$  electrode and kept for incubation at  $4\text{ }^\circ\text{C}$ . In this way, covalent (N–H) bonding between  $\text{NH}_2$  of nanocomposite and COOH of anti-OTA occurred. After the incubation time of 6 h, the electrode was washed with PBS to remove the unbound anti-OTA from the electrode surface. Now in the second step,  $5\text{ }\mu\text{L}$  of the anti-OTA solution was spread over same  $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$  electrode for electrostatic immobilization of anti-OTA on the electrode surface due to the existence of positively charged  $n\text{ZrO}_2$  in the nanocomposite. There was still a chance of the presence of some unspecific active sites on the anti-OTA/ $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$  immunoelectrode surface. To block these unspecific active sites,  $10\text{ }\mu\text{L}$  of BSA ( $0.1\text{ mg mL}^{-1}$ ) was spread over anti-OTA/ $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$  immunoelectrode surface. The fabricated BSA/anti-OTA/ $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$  immunoelectrode was kept at  $4\text{ }^\circ\text{C}$  when not in use. The step-wise fabrication of immunoelectrode is shown in Scheme 1. Different concentrations of OTA from  $1\text{ to }10\text{ ng mL}^{-1}$  were also prepared in PBS of pH 7 to investigate the response studies of BSA/anti-OTA/ $n\text{ZrO}_2\text{-NH}_2\text{C}/\text{ITO}$  immunoelectrode.

### 2.5. Preparation of spiked samples for OTA measurement

The spiked samples for OTA measurement were prepared according

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