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Highly sensitive protein functionalized nanostructured hafnium oxide based biosensing platform for non-invasive oral cancer detection

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ABSTRACT

We report results of the studies relating to the development of a non-invasive, label free immunosensor based on nanostructured hafnium oxide (hafinia) deposited onto indium tin oxide (ITO) coated glass for oral cancer biomarker (CYFRA-21-1) detection in human saliva. The nanostructured hafnia ($nHfO_2$) has been synthesized via one step low temperature hydrothermal process and modified with 3-aminopropyltriethoxy silane (APTES) for covalent immobilization of monoclonal antibodies (anti-CYFRA-21-1). Bovine serum albumin (BSA) was used to block non-specific sites at the anti-CYFRA-21-1/APTES/$nHfO_2$/ITO electrode surface. The structural, morphological and spectroscopic characterization of the synthesized nanomaterials and fabricated electrodes has been carried out using X-ray diffraction (XRD), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FT-IR) and X-ray photoelectron spectroscopy (XPS) studies, respectively. The results of response studies conducted on BSA/anti-CYFRA-21-1/APTES/$nHfO_2$/ITO immunoelectrode reveal that this biosensor has high sensitivity (9.28 $\mu A\,mL\,ng^{-1}\,cm^{-2}$), wide linear detection range (2–18 $ng\,mL^{-1}$) and fast response time (15 min). This immunosensor has been validated with enzyme linked immunosorbent assay (ELISA) in saliva samples of oral cancer patients.

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1. Introduction

Oral cancer occurs due to uncontrolled growth of cells in the mouth and is currently the sixth most common cancer [1]. If undetected at an early stage, this cancer metastasizes in the body leading to death. The conventional methods such as laser capture microdissection, visualization adjuncts, cytopathology and biopsy currently used for detection and monitoring of the oral cancer are time consuming, labor-intensive, expensive and require serum/blood [2–6]. Further, the enzyme linked immunosorbent assay (ELISA) is not user friendly, labor intensive, takes long time and may yield false positives. There is thus an urgent need for the availability of a suitable technique that can be used for rapid detection of oral cancer. In this context, biosensors are considered to be attractive and cost-effective technique that can be used for detection of oral cancer [2,7–10]. Among the various biosensors, electrochemical (EC) biosensors are considered promising since they require small sample volume and are not affected by sample turbidity [4,10–13]. Besides this, these biosensing devices require low-power and can be easily miniaturized [8,14].

The oral cancer detection via identification of biomarkers is considered important. Some of the biomarkers used for oral cancer detection are interleukin-8 (IL-8), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), human epidermal growth factor receptor-2 (HER2), tissue polypeptide antigen (TPA) and epidermal growth factor receptor (EGFR) [4,9,10,15–19]. These biomarkers are found in very low amount ($\sim pg\,mL^{-1}$) in biological fluids. Besides this, these biomarkers are secreted in serum/blood samples and hence the detection is invasive [9–16,20,21]. Detection of oral cancer via salivary biomarker is a promising non-invasive approach. [5,22]. Interstingly, the CYFRA-21-1 antigen is known to be over-secreated in saliva. In normal subjects, the CYFRA-21-1 level is found to be 3.8 $ng\,mL^{-1}$ whereas in oral cancer patients it increases to $17.46 \pm 1.46\,ng\,mL^{-1}$ [22–24]. A noninvasive electrochemical biosensor based on nanostructured zirconia was reported for CYFRA-21-1 detection in saliva samples [25,26].

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The performance of an EC biosensor is known to depend on the physiochemical properties of a given material containing the immobilized biomolecules [8,11,14]. Efforts have been made to immobilize biomolecules onto nanostructured metal oxides [8,27,28]. This is because these nanomaterials exhibit interesting morphological, functional, biocompatible and catalytic properties [27–30] Hafnium (atomic number 72) is a tetravalent transition metal of the IVth group. The $nHfO_2$ is an attractive inorganic metal oxide comprising of hafnium and oxygen. It can be prepared using a one step low temperature hydrothermal process [31,32]. The hafnia is known to have interesting characteristics like high dielectric constant (k), high surface-to-volume ratio, thermal stability, chemical inertness, pH sensitivity, nontoxicity and affinity for groups containing oxygen that make it an interesting material for biosensing application [32–34]. The isoelectric point of hafnia is 7.0 and hence it is surface neutral at physiological pH. The high-k of hafinia is considered advantageous for the reaction between surface immobilized antibodies and the antigen molecules since it may perhaps induce high current changes [25,35]. Furthermore, oxygen moieties in HfO_2 can facilitate covalent attachement of linker molecules that can be useful for immobilization of biomolecules [31,36–38]. The material properties of HfO_2 have recently been investigated for application in semiconductor electronics. Lee et al. proposed an invasive biosensor based on hafnium oxide for detection of human interleukin-10 for cardiovascular disease [37].

This paper contains results of studies relating to the fabrication of $nHfO_2$ based immunosensor based on anti-CYFRA-21-1 for efficient detection of CYFRA-21-1 in saliva samples. Efforts have also been made to investigate the structural and spectroscopic characterization of anti-CYFRA-21-1 immobilized $nHfO_2$ electrode.

2. Experimental details

2.1. Reagents

Hafnium dichloride oxide octahydrate (98+%) ($HfOCl_2{\cdot}8H_2O$), cetyl trimethylammonium bromide (CTAB) ($C_{19}H_{42}BrN$) and 3-aminopropyl triethoxy silane (APTES) ($C_9H_{23}NO_3Si$) were purchased from Alfa Aesar. 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC) ($C_8H_{17}N_3$) of AR grade was purchased from Sigma Aldrich. Sodium hydroxide (NaOH) pellets, sodium monophosphte (NaH_2PO_4), sodium diphosphatedihydrate ($Na_2HPO_4{\cdot}2H_2O$), N-hydroxysulfosuccinimide (NHS) ($C_4H_5NO_3$), sodium chloride (NaCl), potassium ferricyanide $K_3[Fe(CN)_6]$ and potassium ferrocyanide $K_4[Fe(CN)_6]3H_2O$ were purchased from Fisher Scientific. All these chemicals were of analytical grade and were used without any further purification. Phosphate buffer saline (PBS) solution of pH 7.0 was prepared using $Na_2HPO_4{\cdot}2H_2O$ (0.05 mol L^{-1}) and NaH_2PO_4 (0.05 mol L^{-1}). Fresh PBS solution was prepared using Milli-Q water having resistivity of 18.2 MΩ cm and stored at 4 °C. Antigen CYFRA-21-1 and anti-CYFRA-21-1 were purchased from Ray Biotech, Inc., India. These biomolecules were further diluted using PBS buffer of pH 7.0. CYFRA-21-1 ELISA Kit was purchased from Kinesis DX, USA.

2.2. Synthesis of hafnia nanoparticles

Low temperature hydrothermal process was used for the synthesis of hafnia nanoparticles. The solution comprising of 0.04 M of hafnium (IV) dichloride oxide octahydrate and 0.08 M sodium hydroxide was prepared in 70 mL of deionized water. Next, 0.01 M CTAB solution was prepared in 10 mL of deionized water. CTAB solution was added drop-wise in hafnium (IV) dichloride oxide octahydrate solution, after which it was kept for 2 h at 25 °C with constant stirring. Further, sodium hydroxide was added drop-wise in these solutions and kept for stirring for next 2 h under similar conditions. Thus obtained solution contained in teflon was autoclaved and maintained at 170 °C for about 17 h. After cooling, the synthesized material was washed with deionized water until pH of the solution reached 7.0. Next, the whitish slurry was calcinated at 400 °C for 3 h after which it was stored in a cool and dry place until further use. The mechanism of prepration of hafnia nanoparticles is shown in Scheme 1(a).

2.3. Functionalization of hafnia nanoparticles and electrophoretically deposition on ITO electrode (APTES/$nHfO_2$/ITO)

The $nHfO_2$ were functionalized using a low temperature silanization process. 200 mg of $nHfO_2$ dispersed in 50 mL of isopropanol. There after 0.6 g of 98% APTES was added dropwise. Later, 20 mL of deionized water was added and kept for stirring at 300 rpm for 48 h at 50 °C. To remove the unbound APTES molecules, these nanoparticles were washed with deionized water and stored in a dry place.

Indium tin oxide coated glass (ITO) electrode was used as a substrate for fabrication of biosensing platform. The 1 mg mL^{-1} of these functionalized hafnia nanoparticles were dispersed in acetonitrile. Electrophoretic deposition (EPD) technique (Genetix, GX300C instrument) were used for deposition of functionalized nanoparticles. 22 V was applied for 30s for EPD of APTES/$nHfO_2$ onto the pre-hydrolyzed ITO electrode [7]. The optimized surface area of the APTES/$nHfO_2$/ITO electrode was determined to be 0.25 cm^2. This electrode was washed with deionized water and dried at 25 °C.

2.4. Fabrication of BSA/anti-CYFRA-21-1/APTES/$nHfO_2$/ITO immunoelectrode

15 μL of anti-CYFRA-21-1 (50 μg mL^{-1}) was mixed with 7.5 μL of 0.4 M EDC (activator) and 7.5 μL of 0.1 M NHS (coupling agent) for activation of —COOH groups of the antibody molecules. Further, 30 μL of this solution was uniformly spread by drop-casting method onto APTES/$nHfO_2$/ITO electrode. The electrode was kept in a humid chamber at 25 °C for 3 h followed by washing with PBS to remove any unbound antibody molecules. —COOH group of anti-CYFRA-21-1 was covalently bound to —NH_2 terminal of APTES via strong amide bond (OC—NH). Further, bovine serum albumin (BSA = 1 mg dL^{-1}) (20 μL) was used for blocking nonspecific active sites of the anti-CYFRA-21-1/APTES/$nHfO_2$/ITO electrode surface. Later, the BSA/anti-CYFRA-21-1/APTES/$nHfO_2$/ITO immunoelectrode was washed with PBS to remove any unbound BSA. This immunoelectrode was stored at 4 °C under dark conditions until further use. Scheme 1(b) shows a stepwise fabrication process of the BSA/anti-CYFRA-21-1/APTES/$nHfO_2$/ITO immunosensor.

2.5. Collection and processing of saliva samples

Unstimulated whole saliva was collected from ten patients diagnosed with oral cancer. Deionized water (5 mL) was used for rinsing of mouth and expectorated into sterilized tube and kept in ice condition. The collected saliva was centrifuged at 2800 rcf at room temperature (25 °C) for 30 min after which the supernatant was collected in sterilized tube and stored at −20 °C [22]. The saliva samples of oral cancer patient were collected from Rajiv Gandhi Cancer Institute and Research Centre, Delhi (India). All saliva samples were collected under a protocol approved by Rajiv Gandhi Cancer Institute and Research Center Review Board and all the patients provided written informed consent.

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