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

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We report results of the studies relating to the development of a non-invasive, label free immunosensor based on nanostructured hafnium oxide (hafnia) deposited onto indium tin oxide (ITO) coated glass for oral cancer biomarker (CYFRA-21-1) detection in human saliva. The nanostructured hafnia (nHfO2) has been synthesized via one step low temperature hydrothermal process and modified with 3-aminopropyltriethoxysilane (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/nHfO2/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/nHfO2/ITO immunosensor reveal that this biosensor has high sensitivity (9.28 μA 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 (~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]. Interestingly, the CYFRA-21-1 antigen is known to be over-secreted 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 ± 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].
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 [8,27–30]. 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 nHfO2 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, non-toxicity 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 hafnia 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 HfO2 can facilitate covalent attachment of linker molecules that can be useful for immobilization of biomolecules [31–38]. The material properties of HfO2 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 nHfO2 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 nHfO2 electrode.

2. Experimental details

2.1. Reagents

Hafnium dichloride oxide octahydrate (98%+) (HfOCl2·8H2O), cetyl trimethylammonium bromide (CTAB) (C19H32BrN2) and 3-aminopropyl triethoxy silane (APTES) (C3H2NO3Si) were purchased from Alfa Aesar. 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC) (C26H33ClN2) of AR grade was purchased from Sigma Aldrich. Sodium hydroxide (NaOH) pellets, sodium monophosphate (Na2HPO4), sodium diphosphatedihydrated (Na2HPO4·2H2O), N-hydroxysulfo succinimide (NHS) (C6H5NO3), sodium chloride (NaCl), potassium ferricyanide K3[Fe(CN)6] and potassium ferrocyanide K4[Fe(CN)6]·3H2O 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 Na2HPO4·2H2O (0.05 mol L⁻¹) and NaH2PO4 (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 auto-claved 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/nHfO2/ITO)

The nHfO2 were functionalized using a low temperature sialnization process. 200 mg of nHfO2 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 30 s for EPD of APTES/nHfO2 onto the pre-hydrolyzed ITO electrode [7]. The optimized surface area of the APTES/nHfO2/ITO electrode was determined to be 0.25 cm². This electrode was washed with deionized water and dried at 25 °C.

2.4. Fabrication of BSA/anti-CYFRA-21-1/APTES/nHfO2/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/nHfO2/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 –NH2 terminal of APTES via strong amide bond (OC–NH). Further, bovine serum albumin (BSA = 1 mg mL⁻¹) (20 μL) was used for blocking nonspecific active sites of the anti-CYFRA-21-1/APTES/nHfO2/ITO electrode surface. Later, the BSA/anti-CYFRA-21-1/APTES/nHfO2/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/nHfO2/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 2800rcf 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.
2.6. Characterization

Crystallinity and information on phase of the synthesized product were obtained using monochromatic X-ray diffractometer [Bruker D-8 Advance] with Cu-Kα radiation (λ = 1.5406 Å). Morphology and particle size were determined using transmission electron microscopy at an accelerating voltage of 200 kV (Tecnai G2 30 U-twin, Tecnai 300 kV ultratwin microscope). The topography of each APTES/nHfO2/ITO, anti-CYFRA-21-1/APTES/nHfO2/ITO and BSA/anti-CYFRA-21-1/APTES/nHfO2/ITO electrodes was investigated using atomic force microscopy (AFM) on a Park Xe-100 AFM system. X-ray photoelectron spectroscopy (XPS) measurements were performed using Nova, Kratos Analytical Ltd., Manchester, UK. The Fourier transform infrared spectrometer (FT-IR) [PerkinElmer, Spectrum BX II] was used to determine the presence of functional groups and bonds present in APTES/nHfO2/ITO and anti-CYFRA-21-1/APTES/nHfO2/ITO. The electrochemical response studies were conducted using a three-electrode cell via an Autolab Potentiostat (Netherlands). The fabricated electrodes acted as the working electrode, Ag/AgCl as the reference electrode and platinum (Pt) as the counter electrode. ELISA plate reader (iMark, Bio red, USA) was used for determination of CYFRA-21-1 concentration in saliva of oral cancer patients.

3. Results and discussion

3.1. Structural and morphological studies

The results of X-ray diffraction (XRD) studies conducted on nHfO2 are shown in Fig. 1a. The presence of diffraction peaks corresponding to (011), (1–11), (111), (020), (201), (−202), (022), (310) and (−222) planes indicates the formation of a single monoclinic phase (JCPDS 34-0104). The TEM image (Fig. 1b) exhibits a rectangular shape structure arising due to agglomeration (perhaps due to the Brownian motion of the suspended dispersed molecules in solution) and polydispersive nature of HfO2 nanoparticles [39,40].

As reported in literature, the nanoparticles having the same crystallographic orientation are likely to adhere to each other with the highest probability [39,40]. It appears that HfO2 nanoparticles perhaps get linked with each other via Vander waals forces [39,40]. HRTEM image (Fig. 1c) shows oval shaped nanoparticles with an average particle size of 14 nm. Fig. 1d exhibits lattice fringes of the HfO2 nanoparticles. The average lattice spacing obtained as 1.79 Å corresponds to (022) of the crystallographic plane.

The results of surface morphology studies (2-D and 3-D view) conducted on i) APTES/nHfO2/ITO ii) anti-CYFRA-21-1/APTES/nHfO2/ITO and iii) BSA/anti-CYFRA-21-1/APTES/nHfO2/ITO electrodes using atomic force microscopy (AFM) in the tapping mode, are shown in Fig. 2 (A–F). The AFM image of APTES/nHfO2/ITO electrode shows that functionalized nHfO2 are uniformly distributed at the ITO surface resulting in nanoporous structure with an average roughness of ~4.7 nm. After the immobilization of anti-CYFRA-21-1 on APTES/nHfO2/ITO electrode, the average roughness increases to ~7.1 nm. The observed regular globular morphology reveals high loading of the antibody molecules onto APTES/nHfO2 surface via covalent interactions. Further, the BSA/anti-CYFRA-21-1/APTES/nHfO2/ITO electrode reveals an average roughness of ~5.5 nm. The decrease in roughness of the BSA/anti-CYFRA-21-1/APTES/nHfO2/ITO immunoelectrode is due to

Scheme 1. (a) Mechanism for synthesis of hafnia nanoparticles and (b) fabrication steps of BSA/anti-CYFRA-21-1/APTES/nHfO2/ITO platform for oral cancer detection.
to the presence of BSA molecules that block non-specific binding sites at the immuno-electrode surface.

3.2. X-ray photoelectron spectroscopy (XPS) studies

Fig. 3 shows the wide scan X-ray photoelectron spectra obtained for (a) nHfO₂ and (b) APTES/nHfO₂. In both spectra, the characteristic peaks observed at 16.0, 212.5, 223.2 and 530.1 eV are assigned to Hf 4f7/2, Hf 4d5/2, Hf 4d3/2 and O 1s, respectively [27,41]. In spectrum (b), additional peaks are observed at 101.6, 285.1 and 399.4 eV due to the presence of Si, C 1s and N 1s, respectively revealing functionalization of nHfO₂ with APTES [27,41]. Further, XPS spectra of hafnium 4f, oxygen 1s and nitrogen 1s regions of nHfO₂ and APTES/nHfO₂ are deconvoluted into characteristic binding energy peaks using Shirley-type baseline and Lorentzian-Doniac-Sunscic curves, with a Gaussian profile as shown in Fig. 3(c–g) [27]. In Fig. 3(e and f), the peak observed at 16.0 eV indicates presence of hafnium (4f) and the peak seen at 17.5 eV reveals presence of the bond between hafnium and oxygen molecules. Fig. 3(c and d), the peak observed near 529.5 eV indicates O₂– states of nHfO₂ while the peak seen at 531 eV is assigned to the presence of C–O, C=O [Fig. 3(d and f)] [41]. Fig. 3(g) shows the N 1s spectra wherein a typical binding energy peak found at 398.5 eV is due to N 1s electrons whereas the peak seen at 399.4 eV is attributed to the presence of nitrogen in free amino groups of APTES molecules [27,42]. These results indicate functionalization of nHfO₂ with APTES molecules that can be used for covalent immobilization of –COOH containing biomolecules.

3.3. Fourier transformed infra-red spectroscopic (FT-IR) studies

FT-IR spectra obtained for the APTES/nHfO₂/ITO and anti-CYFRA-21-1/APTES/nHfO₂/ITO electrode are shown in Fig. 4(a) and (b), respectively. Both the spectra show peak at 495 cm⁻¹ indicating presence of Hf–O bond [43]. The FT-IR spectra of APTES/nHfO₂/ITO electrode shows intense peak at 1561 cm⁻¹ corresponding to –NH₂ groups on the free surface. The peak seen at 2567 cm⁻¹ arises due to the presence of C–H bond present in the APTES molecules with sp³ hybridization. A broad peak present between 2900 and 3400 cm⁻¹ is due to immersion of the N–H stretching vibration with –OH stretching of water molecules [27]. Further, FT-IR spectra of anti-CYFRA-21-1/APTES/nHfO₂/ITO electrode exhibits peaks at 1600 and 1746 cm⁻¹ that are due to the presence of C–N stretching of amide bond II indicating immobilization of anti-CYFRA-21-1 at the APTES/nHfO₂/ITO electrode. [27]

3.4. Electrochemical studies

3.4.1. Electrochemical impedance spectroscopy (EIS) studies

EIS studies were conducted to investigate the interfacial properties between the electrode and electrolyte, adsortion behavior and interactions of biomolecules with the electrode surface. The Nyquist plot represents the real part of impedance at X-axis and imaginary part at Y-axis. This Nyquist plot can be modelled by an equivalent circuit (Randles circuit) comprising of the solution resistance (Rₛ), charge transfer resistance (Rₓ), Warburg impedance (W) and double layer capacitance (Cₓ) etc. Nyquist plot includes a semicircle region observed at higher frequency corresponding to electron-transfer limited process and is followed by a linear straight line at 45° to the real axis at lower frequencies, revealing diffusion limited electron transfer process. The semicircle diameter of EIS spectra gives value of R_xt which reveals electron transfer kinetics of redox probe at the electrode interface. Moreover, Rs and Warburg impedance (Zw) representing bulk properties of the electrolyte solution and diffusion of applied redox probe respectively. Fig. S1 shows Nyquist plots obtained for APTES/nHfO₂/ITO, anti-CYFRA-21-1/APTES/nHfO₂/ITO and BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO electrode carried out at a biasing potential of 10 mV in the frequency range 100 KHz to 0.1 Hz using PBS (50 mM, 0.9% NaCl) with [Fe(CN)₆]₃⁻/₄⁻ (5 mM). The Rₓ value of 2.3 ± 0.4 kΩ for of APTES/nHfO₂/ITO electrode.
Fig. 2. 2D and 3D AFM images of (A-B) APTES/nHfO₂/ITO (C-D) anti-CYFRA-21-1/APTES/nHfO₂/ITO and (E-F) BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO.

decreases (1.2 ± 0.5 KΩ) after immobilization of the anti-CYFRA-21-1. This is due to presence of the favourable microenvironment and spatial orientation of the antibody molecules that facilitate increased charge transfer process. Further, after BSA immobilization Rct value increases to 1.9 KΩ due to both macromolecular structure and insulating nature that hinders the charge transfer.

The heterogeneous electron transfer rate constants (K₀) of APTES/nHfO₂/ITO and anti-CYFRA-21-1/APTES/nHfO₂/ITO electrodes were determined using Eq. (1):

$$K_0 = \frac{RT(n^2F^2AR_{ct}C)}{1}$$
where $R$ is the gas constant, $T$ is the temperature, $n$ is the electron transfer constant of the redox couple, $F$ is Faraday constant, $A$ is the effective area of the electrode, $C$ is the concentration of redox couple in the bulk solution. It is found that the value of $K_0$ of APTES/nHfO$_2$/ITO obtained as $23.6 \times 10^{-6}$ cm$^{-1}$S increases to $43.4 \times 10^{-6}$ cm$^{-1}$S after antibody functionalization. This indicates enhanced electron transfer kinetics of the immunoelectrode due to favourable spatial orientation. Further in case of the BSA/anti-CYFRA-21-1/APTES/nHfO$_2$/ITO electrode, $K_0$ decreases to $27.7 \times 10^{-6}$ cm$^{-1}$S due to macromolecular structure and insulating nature of BSA that hinders the electron transfer.

3.4.2. Cyclic voltammetry studies

The effect of pH on the electrochemical behaviour of the fabricated immunoelectrode (BSA/anti-CYFRA-21-1/APTES/nHfO$_2$/ITO) was investigated using cyclic voltammetry (CV) in PBS (50 mM, 0.9% NaCl) buffer at pH 6.0, 6.5, 7.0, 7.4, 8.0, with [Fe(CN)$_6$]$^{3-}/^{4-}$ (5 mM) at scan rate of 50 mV/s in the potential range −0.8 to 0.8 V. It is found that this electrode exhibits maximum current at pH 7.0 (Fig. S2). This may be due to the fact that biological molecules (e.g. amino acid, enzyme, antigen, antibody etc.) are present in natural form with high activity at neutral pH. In the acidic or basic medium, the antibodies get denatured due to the presence of H$^+$ or OH$^-$ ions [7,44]. Therefore, further electrochemical measurements were performed in buffer at pH 7.0. The CV of each ITO, APTES/nHfO$_2$/ITO, anti-CYFRA-21-1/APTES/nHfO$_2$/ITO and BSA/anti-CYFRA-21-1/APTES/nHfO$_2$/ITO electrodes was recorded respectively (Fig. S3). The anodic peak current of ITO electrode ($I_{pa}=0.40$ mA) was found to decrease in case of the APTES/nHfO$_2$/ITO electrode ($I_{pa}=0.18$ mA). However, after the anti-CYFRA-21-1 immobilization the peak current increased to 0.32 mA. This increase in peak current is perhaps...
due to availability of the non-binding sites (i.e. free NH₃⁺ group) onto the anti-CYFRA-21-1/APTES/nHfO₂/ITO electrode resulting in accelerated electron transfer between anti-CYFRA-21-1 and the APTES/nHfO₂/ITO electrode [31,37]. The peak current of BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO immunoelectrode (Ipa = 0.30 mA) decreases due to blocking of non-specific active sites by BSA molecules present at the immunoelectrode surface, resulting in hindered electron transfer between solution and the electrode.

The response studies were conducted on the APTES/nHfO₂/ITO electrode and BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO immunoelectrode as a function of scan rate in the range of 40–150 mV/s as shown in Fig. S4 and S5, respectively. The peak-to-peak separation in case of both the electrodes increases and shifts toward the higher potential side with increasing scan rate leading to diffusion controlled (quasi-reversible) process [7,29]. The inset A in Fig. S4 and S5 shows the variation of anodic (Ipa) and cathodic (Ipc) peak current versus square root of scan rate. The linear curve fitting gives the following equations:

\[
I_{pa}(\text{APTES/nHfO}_2/\text{ITO}) = [18.2 \mu A(s/mV) \times \text{scanrate[mV/s]}^{1/2}]
+ 48.97 \mu A, R^2 = 0.999
\]  
(2)

\[
I_{pc}(\text{APTES/nHfO}_2/\text{ITO}) = -[17.52 \mu A(s/mV) \times \text{scanrate[mV/s]}^{1/2}]
- 39.15 \mu A, R^2 = 0.999
\]  
(3)

\[
I_{pa}(\text{BSA/anti-CYFRA-21-1/APTES/nHfO}_2/\text{ITO}) = [44.17 \mu A(s/mV) \times \text{scanrate[mV/s]}^{1/2}]
+ 21.71 \mu A, R^2 = 0.999
\]  
(4)

\[
I_{pc}(\text{BSA/anti-CYFRA-21-1/APTES/nHfO}_2/\text{ITO}) = -[35.10 \mu A(s/mV) \times \text{scanrate[mV/s]}^{1/2}]
- 84.10 \mu A, R^2 = 0.998
\]  
(5)

Inset B in Fig. S4 and S5 shows plot between the potential peak shift (\(\Delta V = V_p - V_{pc}\), \(V_{pa}\) is anodic peak potential and \(V_{pc}\) is cathodic peak potential) and square root of the scan rate obtained for APTES/nHfO₂/ITO and BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO electrode, respectively. The linear curve fitting gives the following equations:

\[
V_{\text{APTES/nHfO}_2/\text{ITO}} = [0.042 V(s/mV) \times \text{scanrate[mV/s]}^{1/2}]
+ 0.17 V, R^2 = 0.997
\]  
(6)

\[
V_{\text{BSA/anti-CYFRA-21-1/APTES/nHfO}_2/\text{ITO}} = [0.031 V(s/mV) \times \text{scanrate[mV/s]}^{1/2}]
+ 0.53 V, R^2 = 0.997
\]  
(7)

These linear relationships indicate that electrochemical reactions are diffusion controlled (quasi-reversible) [29]. The diffusion coefficient (or diffusivity) of the redox species [Fe(CN)₆]³⁻/⁴⁻ was estimated using Randles-Sevick equation:

\[
I_p = \frac{(2.69 \times 10^5)n^{3/2}A^{1/2}D^{1/2}v^{1/2}}{T}
\]  
(8)

where \(I_p\) is the peak current of the immuno electrode, \(n\) is the number of electrons transferred (1), \(A\) is surface area of the electrode (0.25 cm²), \(D\) is diffusion coefficient, \(C\) is the concentration of redox species (5 × 10⁻⁵ mol cm⁻²) and \(v\) is the scan rate (50 mVs⁻¹). The value of diffusion coefficient has been obtained as 2.12 × 10⁻⁹ cm² s⁻¹. The surface concentration of BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO immunoelectrode estimated through Brown-Anson model as given in Eq. (9):

\[
I_p = n^2F^2γAt(4RT)^{-1}
\]  
(9)

where \(I_p\) represents the peak current, \(A\) is the surface area of the electrode, \(v\) is the scan rate (V/s), \(γ\) is the surface concentration of the absorbed electro-active species, \(F\) is the Faraday constant (96485 C mol⁻¹), \(R\) is the gas constant (8.314 J mol⁻¹ K⁻¹) and \(T\) is room temperature (25°C or 298 K). The surface concentration of BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO, is estimated to be as 2.87 × 10⁻⁹ mol cm⁻² [45,46].

3.4.3. Electrochemical response studies

The electrochemical response of BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO immunoelectrode determined as a function of CYFRA-21-1 concentration (2–18 ng mL⁻¹) is shown in Fig. 5. The experiments were conducted in PBS buffer (50 mM, 0.9% NaCl) containing [Fe(CN)₆]³⁻/⁴⁻ (5 mM) at a scan rate of 50 mV s⁻¹.

Fig. 4. FTIR spectra of (a) APTES/nHfO₂/ITO and (b) anti-CYFRA-21-1/APTES/nHfO₂/ITO.
in the potential range, –0.8 to 0.8 V using CV technique. The immunoelectrode was incubated with antigen solution for 15 min for antigen-antibody interaction prior to the CV measurements. It was found that the electrochemical peak current gradually decreased linearly with increased concentration of CYFRA-21-1 (inset b in Fig. 5). The decreased current is attributed to the formation of electrically insulating antigen-antibody complex that perhaps obstructs the electron transfer through \([\text{Fe(CN)}_6]^{3-\text{4+}}\) redox conversion. The observed calibration curve between peak current and antigen concentration obeys Eq. (10)

\[
I_p = \frac{2.32 (\mu \text{A mL g}^{-1}) \times \text{concentration of CYFRA-21-1 (ng mL}^{-1})}{0.334 \mu \text{A}},
\]

\[
R^2 = 0.988
\]

(10)

Fig. 5 (inset b) reveals that linearity is obtained in the range, 2–18 ng mL\(^{-1}\), sensitivity is 9.28 μA mL g\(^{-1}\) cm\(^{-2}\) with regression coefficient \((R^2)\) of 0.983 and the lower detection limit is 0.21 ng mL\(^{-1}\). The limit of detection can be calculated using the standard Eq. (11):

Limit of detection = 3σ/Sensitivity

\[
\text{where } \sigma \text{ is the standard deviation of the BSA/anti-CYFRA-21-1/APTES/nHfO}_2/\text{ITO immunoelectrode. This fabricated immunoelectrode can be used up to 30 times after which the peak current rapidly decreases. The storage stability of the BSA/anti-CYFRA-21-1/APTES/nHfO}_2/\text{ITO immunoelectrode can be determined by recording CV at regular intervals of time up to 10 weeks. The immunoelectrode was stored at 4 °C when not in use. This bioelectrode was found to exhibit 95% of response up to about 8 weeks after which the peak current value abruptly decreased (Fig. S6). Further, we investigated reproducibility of six different fabricated immunoelectrodes (BSA/anti-CYFRA-21-1/APTES/nHfO}_2/\text{ITO) under similar conditions and the variation of electrochemical response as shown in Fig. S7. It was found that the immunoelectrodes showed high reproducibility with relative standard deviation of 1.53%.}

3.4.4. Interferent and control studies

The BSA/anti-CYFRA-21-1/APTES/nHfO}_2/\text{ITO immunoelectrode was pre-incubated with different interfering species present in human saliva such as carcino embryonic antigen (CEA) (concentration 4–16 ng mL\(^{-1}\), cardiac troponin I (Tn-I) (concentration: 0.19 ng mL\(^{-1}\)), sodium carboxymethyl cellulose (NaCM) (concentration: 10 g L\(^{-1}\)) and glucose (7 mg mL\(^{-1}\)). The observed current response of immunoelectrode in the presence of these interferents is shown in Fig. S8. No significant change in current was observed due to addition of the interfering species. On the other hand, the peak current decreased on addition of CYFRA-21-1 (2 ng mL\(^{-1}\)) indicating that the BSA/anti-CYFRA-21-1/APTES/nHfO}_2/\text{ITO immunoelectrode specifically interacted with CYFRA-21-1 and the response was not affected due to the presence of potential interferents present in saliva.

A control experiment conducted using the APTES/nHfO}_2/\text{ITO electrode under similar conditions (Fig. S9) did not exhibit any significant change in the current response of the electrode with increasing concentration of CYFRA-21-1. These results revealed that APTES/nHfO}_2/\text{ITO electrode did not interact with CYFRA-21-1 molecules in absence of antibodies.}

3.4.5. Real sample analysis

We obtained saliva samples of ten oral cancer patients and three normal subjects for determination of CYFRA-21-1 antigen. Samples were processed as described earlier and analyzed through standard enzyme linked immuno sorbent assay (ELISA) techniques. We quantified the concentration of CYFRA-21-1 through double-antibody sandwich ELISA kit (Kinesis DX, USA) in triplicate. The microtiter wells were pre-coated with anti-CYFRA-21-1. After following all the steps, colorimetric reaction occurred and the absorbance was recorded at 450 nm in ELISA plate reader. A series of CYFRA-21-1 concentration in saliva samples obtained by ELISA were used to test accuracy of the fabricated biosensor. The current response recorded for these real samples, matched with the current obtained for standard samples of the same concentration. We found excellent correlation between the current obtained for real sample and standard sample concentrations (Fig. 6 and S10, Table S1 and S2). The observed results revealed less than 10% of %RSD (relative standard deviation) indicating high accuracy of the fabricated biosensor. Table 1 shows characteristics of the BSA/anti-CYFRA-21-1/APTES/nHfO}_2/\text{ITO based biosensor alongwith those reported in literature for oral cancer detection. The sensitivity of the nHfO}_2 based biosensor was found to be 9.28 μA mL g\(^{-1}\) cm\(^{-2}\) which is higher as compared to nZrO}_2 (2.2 μA mL g\(^{-1}\) cm\(^{-2}\)) and nZrO}_2/RGO (0.76 μA mL g\(^{-1}\) cm\(^{-2}\)) based biosensors. Further the response...
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4. Conclusion

nHFO2 has been synthesized via hydrothermal method and functionalized through APTES. The APTES/nHFO2/ITO has been fabricated using electrophoretic deposition method that has been biofunctionalized with the antibodies. The results of the studies conducted on the BSA/anti-CYFRA-21-1/APTES/nHFO2/ITO electrode have revealed good linearity in the range, 2–18 ng mL−1 (with regression coefficient = 0.988), high sensitivity 9.28 µA mL ng−1 cm−2, and lower detection limit of 0.21 ng mL−1. The shelf life of the immunoelectrode is 8 weeks and this electrode can be used up to 30 times. This label free, non invasive BSA/anti-CYFRA-21-1/APTES/nHFO2/ITO immunosensor has been used for detection of oral cancer in clinical samples. Efforts should be made to investigate the effect of orientation arising due to biomolecules (e.g. protein A or G, NHS-LC-biotin, cysteine, histidine, lysine) on the sensing characteristics of this biosensor and to utilize the nHFO2 based smart platform for detection of other cancer biomarkers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2016.05.047.

References


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