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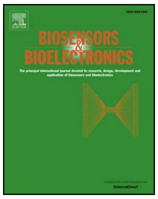

Effect of Brownian motion on reduced agglomeration of nanostructured metal oxide towards development of efficient cancer biosensor

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ABSTRACT

We report results of the studies relating to fabrication of nanostructured metal oxide (NMO) based cancer biosensor. With the help of 2D electroactive reduced graphene oxide (RGO), we successfully inhibited the Brownian motion of NMO that led to reduced agglomeration of NMO. The nanostructured hafnium oxide ($nHfO_2$) was used as a model NMO. The reduced agglomeration of $nHfO_2$ was achieved through controlled hydrothermal synthesis and investigated via nanoparticles tracking analysis (NTA). X-ray diffraction (XRD), scanning electron microscopy (SEM) and transmission electron microscope (TEM) techniques were used for phase identification as well as morphological analysis of the synthesized nanohybrid ($nHfO_2$@RGO) material. The 3-aminopropyl triethoxysilane (APTES) was used for the functionalization of $nHfO_2$@RGO and electrophoretic deposition (EPD) technique was used for its deposition onto ITO coated glass electrode. Further, antibodies of cancer biomarker (anti-CYFRA-21-1) were immobilized via EDC-NHS chemistry and Bovine serum albumin (BSA) was used for blocking of the non-specific binding sites. The electrochemical response studies of fabricated immunoelectrode (BSA/anti-CYFRA-21-1/APTES/$nHfO_2$@RGO/ITO) revealed higher sensitivity (18.24 $\mu A\ mL\ ng^{-1}$), wide linear detection range (0 to 30 $ng\ mL^{-1}$), with remarkable lower detection limit (0.16 $ng\ mL^{-1}$). The obtained results showed good agreement with the concentration of CYFRA-21-1 obtained through enzyme linked immunosorbent assay (ELISA) in saliva samples of oral cancer patients.

1. Introduction

Nanostructured metal oxides (NMOs) owing to their unique physical, chemical and electronic properties have recently drawn much interest towards the fabrication of various biosensing platforms (Luo et al., 2006; Solanki et al., 2011). These materials provide high surface-to-volume ratio, surface reaction activity, chemical, thermal and mechanical stability, high catalytic efficiency as well as strong adsorption ability that can facilitate immobilization of biomolecules with desired orientation (Malhotra et al., 2012; Solanki et al., 2009). A number of metal oxides including zinc oxide, iron oxide, titanium oxide, zirconium oxide, magnesium oxide, hafnium oxide etc. have been used for application in biosensors (Das et al., 2011; Kumar et al., 2016c; Lee et al., 2012; Malhotra et al., 2012; Solanki et al., 2011). Among the various metal oxides, nanostructured hafnium oxide ($nHfO_2$) has recently aroused considerable interest for the fabrication of biosensing platforms. This NMO exhibits high dielectric constant ($20-25$), large surface-to-volume ratio, thermal stability, non-toxicity, pH sensitivity with neutral isoelectric point (7.0) and is thus a promising candidate for fabrication of desired biosensing platform (Chen et al., 2010; Fahrenkopf et al., 2012; Lee et al., 2012). Further, the presence of oxygen moieties in $nHfO_2$ facilitates covalent conjugation of linker molecules (APTES) with hydrolyzed ITO electrode (Fahrenkopf et al., 2012; Shim et al., 2013). It has been predicted that Brownian motion of the suspended dispersed $nHfO_2$ in solution may result in agglomeration resulting in the formation of large cluster molecules with low surface-to-volume ratio that may influence the biosensing parameters.(Eliziário et al., 2009; Lee et al., 2006) However, this problem may perhaps be overcome by inhibiting Brownian motion in $nHfO_2$ by providing supporting matrix that has high surface area, large number of electroactive sites with conducting behavior.

The chemically reduced graphene oxide is an organic material and is a promising substrate for uniform distribution of NMOs due to its rich defects and electroactive sites that may prevent the agglomeration of

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nanosized molecules. Besides this, the reduced chemical groups may facilitate charge transfer kinetics leading to enhanced electrochemical properties that may provide both covalent and non-covalent sites for surface functionalization resulting in improved biomolecules loading with uniform conducting surface (Ali et al., 2016; Ibrahim et al., 2016; Teymourian et al., 2014; Yang et al., 2016).

RGO is known to have excellent electrochemical conductivity, rapid heterogeneous electron transfer rate (HET), superior mechanical flexibility and remarkable stability that may assist the fabrication of efficient biosensing platform (Ali et al., 2016; Teymourian et al., 2014; Yang et al., 2016). Integration of RGO with NMOs may produce synergistic effect resulting in enhanced conductivity, stability, better catalytic behavior and improved charge transfer kinetics (Teymourian et al., 2014; Xu et al., 2013). It has been found that RGO incorporated NMOs can provide a stable nanohybrid through mutual electrostatic interactions between the positively charged NMOs and negatively charged RGO sheet preventing agglomeration of NMOs onto RGO sheet resulting in improved electrochemical properties (Ali et al., 2016). Teymourian et al. fabricated electrochemical biosensors based on nanocomposite of zirconia decorated reduced graphene oxide wherein improved electrochemical performance was found for different electroactive compounds (Teymourian et al., 2013). Yang et al. demonstrated RGO/Fe_3O_4/CdSe nanocomposite based chemiluminescence immunosensor for interleukin-6 detection. Xie et al. reported Co_3O_4-RGO nanocomposite based biosensor for glucose detection that showed wide linear range (Xie et al., 2013).

Oral cancer (OC) has become the 6th most common cancer in the world and is known to occur more often in men than women. The OC if not detected at an early stage, may metastasize to other parts of body leading to death. Conventional techniques such as brush cytological tests, laser capture microdissection, biopsy and toluidine blue staining are currently being used for diagnosis of oral cancer (Kumar et al., 2015). However, these methods are invasive and highly painful as they require tissue sample. Moreover, these methods are labor intensive, expensive, time consuming and require skilled personnel for specimen collection and analysis. Thus, demand for a non-invasive biosensing technique for rapid and early detection of OC is increasing. In this context, saliva has been used as an efficient biological medium for its detection. Biologically it is similar to blood sample because level of biomolecules changes with the progression of disease (Kumar et al., 2015).

Saliva has various advantages over blood such as collection is non-invasive, painless sample collection, easy handling and for storage it does not require anticoagulant (de Almeida et al., 2008; Giannobile et al., 2009; Kumar et al., 2016c). In saliva sample of OC patients several biomolecules such as sialic acid, CD-59, TNF-α, CA-125, miR-125a, miR-200a, IL-1, IL-6, IL-8 etc. are secreted in low concentration (Cheng et al., 2014; Hu et al., 2008; Kumar et al., 2013; Lee and Wong, 2009; Markopoulos et al., 2010; Park et al., 2009). Recently for non-invasive detection of OC, CYFRA-21-1 biomarker has been found to play an important role due to its higher secretion as well as a specific cut off value (Malhotra et al., 2016; Rajkumar et al., 2015). Efforts have been made to detect CYFRA-21-1 biomarker in saliva. Kumar et al. reported electrochemical biosensors based on $nZrO_2$ and its composite, $nHfO_2$ for oral cancer biomarker detection. These biosensors were found to have inferior biosensing parameters (Kumar et al., 2016a, 2015, 2016b, 2016c; Tiwari et al., 2016). To overcome these limitations, reduced the agglomeration of NMO by controlling its Brownian motion, may perhaps play an important role.

We report for the first time the facile one pot *in situ* synthesis of $nHfO_2$@RGO nanohybrid towards reduced agglomeration of $nHfO_2$ and its application in development of cancer biosensor. As compared to that based on agglomerated $nHfO_2$, the synthesized non-agglomerated $nHfO_2$ onto RGO based immunosensor exhibited eleven times more HET rate and have superior biosensing parameters.

2. Experimental section

2.1. Fabrication of immunosensing platform

To fabricate BSA/anti-CYFRA-21-1/APTES/$nHfO_2$@RGO/ITO immunoelectrode, the functionalized $nHfO_2$@RGO nanohybrid (APTES/$nHfO_2$@RGO) was deposited onto a pre-hydrolyzed indium tin oxide (ITO) coated glass substrate via EPD technique. The ITO was used as the working electrode, platinum wire as a reference electrode. Both electrodes were separated by 1 cm in the glass cell. Further, 25 mg of APTES/$nHfO_2$@RGO was dispersed in 25 mL of acetonitrile to form the colloidal suspension. An optimized DC potential (35 V) was applied for 60 s to obtain uniform film of APTES/$nHfO_2$@RGO onto ITO electrode. Next, a solution containing 15 μL of anti-CYFRA-21-1 (50 $\mu g\ mL^{-1}$), 7.5 μL of EDC (0.2 M) and 7.5 μL of NHS (0.05 M) was prepared, where EDC acted as coupling agent and NHS for the activation of -COOH group present on anti-CYFRA-21-1 molecules. 30 μL of activated anti-CYFRA-21-1 (50 $\mu g\ mL^{-1}$) solution was drop cast over APTES/$nHfO_2$@RGO/ITO electrode and was kept in a humid chamber for about 3 h. After incubation the electrode was washed with PBS solution (pH = 7.0) to remove any unbound antibody molecules. Further, for blocking of the non-specific active sites, 30 μL of freshly prepared BSA (1 $mg\ mL^{-1}$) was used. The fabricated BSA/anti-CYFRA-21-1/APTES/$nHfO_2$@RGO/ITO immunoelectrode was further washed with PBS solution and stored at 4 °C till further use. Scheme S1 shows the schematic representation of immunoelectrode fabrication.

3. Results and discussion

3.1. Structural and morphological studies

Fig. 1a shows the XRD pattern of synthesized $nHfO_2$@RGO nanohybrid in the 2θ range 20° to 80°. The peak at 24.4° corresponding to (002) plane reveals the conversion of GO to RGO through hydrothermal process.(Srivastava et al., 2013) The other peaks obtained at 2θ angle of 28.5°, 31.6°, 34.7°, 38.9°, 41.1°, 45.3°, 50.3°, 55.7°, 61.7°, 65.8°, 71.4°, 75.6° and 77.9° correspond to (−111), (111), (020), (021), (−112), (211), (022), (310), (311), (−231), (123), (140) and (330) planes, respectively indicating the formation of monoclinic phase of $nHfO_2$ (JCPDS No. 340104). No additional peak was observed indicating the synthesis of pure monoclinic phase of $nHfO_2$ with RGO sheet. The crystalline size of hafnium oxide was calculated as 10 nm for the (020) plane using Scherrer formula (Eq. (1)) indicating the presence of hafnium oxide crystallites grown along the (020) direction.

$$D = \frac{0.94\lambda}{\beta Cos\theta} \tag{1}$$

where D is the crystalline size of the nanomaterial, λ is the X-ray wavelength (1.540 Å), β is the full width at half maxima and θ is the Bragg's angle.

The size of synthesized nanoparticles plays an important role in the efficiency of the biosensing parameters. Kumar et al. (2016b) synthesized $nHfO_2$ nanoparticles via hydrothermal process. During this, the nanoparticles undergo Brownian motion due to their random collision leading to the agglomeration and formation of larger particles (~120 nm) whereas; the individual size of the synthesized $nHfO_2$ was ~14 nm (Fig. S1). Brownian motion of the particle is known to be directly proportional to the diffusion coefficient (D) of the particle which can be calculated by the Stoke Einstein equation (Eq. (2)):

$$D = \frac{K_B T}{6\pi\eta r} \tag{2}$$

where K_B is Boltzmann constant (1.38×10^{-23} J/K), T is temperature, η is dynamic viscosity of the solvent and r is hydrodynamic radius of the particle. The diffusion coefficient of $nHfO_2$ (individual particle size of $nHfO_2 = 14$ nm) was calculated to be $1.75 \times 10^{-9}\ m^2/s$ due to the