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# Nanostructured zirconia decorated reduced graphene oxide based efficient biosensing platform for non-invasive oral cancer detection

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ABSTRACT

We report results of the studies relating to fabrication of a non-invasive, label-free and an efficient biosensing platform for detection of the oral cancer biomarker (CYFRA-21-1). One step hydrothermal process was used for uniform decoration of nanostructured zirconia (average particle size 13 nm) on reduced graphene oxide ($ZrO_2$–RGO) to avoid coagulation of the zirconia nanoparticles and to obtain enhanced electrochemical performance of $ZrO_2$–RGO nanocomposite based biosensor. Further, $ZrO_2$–RGO has been functionalized using 3-aminopropyl triethoxy saline (APTES) and electrophoretically deposited on the indium tin oxide coated glass substrate at a low DC potential.The APTES/$ZrO_2$–RGO/ITO electrode exhibits improved heterogeneous electron transfer (more than two times) with respect to that of the APTES/$ZrO_2$/ITO electrode indicating faster electron transfer kinetics. The –$NH_2$ containing APTES/$ZrO_2$–RGO/ITO platform is further biofunctionalized with anti-CYFRA-21-1. The structural and morphological investigations of the $ZrO_2$–RGO based biosensing platform have been accomplished using X-ray diffraction (XRD), electrochemical, transmission electron microscopy (TEM), atomic force microscopy (AFM) and Fourier transform infrared spectroscopy (FT-IR) studies. This immunosensor exhibits a wider linear detection range (2–22 ng $mL^{-1}$), excellent sensitivity (0.756 μA mL $ng^{-1}$) and a remarkable lower detection limit of 0.122 ng $mL^{-1}$. The observed results have been validated via enzyme linked immunosorbent assay (ELISA).



## 1. Introduction

Non-invasive detection strategy is considered to be a promising alternative for detection of human diseases (Guilbault et al., 1995). To accomplish this, saliva, tear, urine and sweat have been proposed as interesting for desired biomedical investigations (Chen et al., 2011; Tille, 2013). Among these, saliva based clinical testing has many advantages due to easier saliva collection, low cost of storage and easy transport (Lawrence, 2002). Further, the non-invasive saliva based collection techniques can dramatically reduce anxiety and discomfort for monitoring disease and general health of a patient. Saliva is a useful non-invasive body fluid that can be used for biomarker detection. Besides this, it is one of the simplest and easily accessible body fluid that can be utilized for a wide range of applications including early detection and monitoring of diseases including oral cancer (Lawrence, 2002; Tille, 2013).

Oral cancer (OC) is currently the sixth most common cancer and is caused by uncontrolled growth of cells in the floor of mouth, lips, sinuses, tongue, cheeks, throat etc. (Kujan et al., 2006; Kumar et al., 2015a). The main reasons behind this cancer are chewing of tobacco, smoking, alcohol consumption, gastro-esophageal reflux disease, human papillomavirus and exposure to chemicals (e.g. formaldehyde and asbestos) (Gorschinski et al., 2009). These high risk factors may alter the expression of p16, APC and p53 genes resulting in the origin of oral cancer (Gonzalez et al., 1997). This cancer can be life threatening if not detected and is not treated at an early stage. For diagnosis of the oral cancer, laser-capture micro-dissection, visualization adjuncts, cytopathology and biopsy techniques can be used and these techniques require tissue specimens that are highly painful to obtain (Mehrotra and Gupta, 2011; Patton et al., 2008; Scully et al., 2008). Besides this, these methods are highly expensive, time consuming and require skilled personnel for specimen collection. However, the non-invasive biosensing is attractive, cost effective, accurate and easier to use for oral cancer detection (Malhotra et al., 2012; Mehrotra and Gupta, 2011; Scully et al., 2008).

The salivary biomarkers such as Interleukin-8 (IL-8), Interleukin-6 (IL-6), Interleukin-1 (IL-1), Interleukin-1β (IL-1β), TNFα-1, Endotheline-1 (ET-1), HNP-1, hsa-miR-200a can be used for early diagnosis and prognosis of the oral cancer (Malhotra et al., 2010; Pickering et al., 2007; Punyani and Sathawane, 2013). However,

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only a few reports are available for detection of salivary biomarkers using biosensing technique. Most of these biomarkers are secreted in very low amount (pg $mL^{-1}$) (Malhotra et al., 2012, 2010; Mehrotra and Gupta, 2011). CYFRA-21-1, a proteinaceous biomarker (also known as cytokeratin-19) is a member of keratin family that is known to maintain structural integrity of the epithelial cells. It is a 40 kDa molecule encoded by *KRT19* gene. In saliva of oral cancer patients, it is secreted in higher concentration ($17.46 \pm 1.46$ ng $mL^{-1}$) (Nagler et al., 2006; Rajkumar et al., 2015).

Transducers are known to play an important role towards the fabrication of an efficient biosensor. It provides an interactive platform for the immobilization of biomolecules on desired substrate and can be used for monitoring of an electrical signal resulting due to an electrochemical reaction at an electrode surface, usually as a result of an imposed potential, current or frequency. The unique properties of a transducer can be helpful in improving the characteristics of a biosensor (Roushani and Valipour, 2016; Solanki et al., 2011). Nanostructured metal oxides (NMOs) have been found to have interesting optical and electrical properties due to electron and phonon confinement, high surface-to-volume ratio, modified surface work function, high surface reaction activity, high catalytic efficiency and strong adsorption ability. The NMOs can thus be utilized for increased loading of desired biomolecules per unit mass of particles. Among the various NMOs, zirconia has been found to have interesting physiochemical as well as biosensing characteristics (Das et al., 2011). Biocompatibility, excellent electrical and surface charge properties, oxygen moieties in $ZrO_2$ make it a promising material as a transducer in the fabrication of a biosensor (Das et al., 2011; Liu and Lin, 2005; Kumar et al., 2015a). In this context, it has been reported that zirconia nanoparticles tend to aggregate and form large clusters (Kumar et al., 2015a; Zhao et al., 2006). To overcome these problems, a substrate material with a high surface area and good conductivity is crucial for increasing the physiochemical and biosensing activity of zirconia nanoparticles.

The chemically reduced graphene oxide (GO) has recently been found to be a promising substrate for uniform distribution of metal oxide nanoparticles (Dong et al., 2012; Sawangphruk et al., 2013; Wei et al., 2011). The reduced graphene oxide (RGO) has been demonstrated to have excellent electrochemical conductivity due to rapid heterogeneous electron transport (HET), superior mechanical flexibility and remarkable stability that can be helpful for the fabrication of an efficient biosensing platform (Ali et al., 2014). Gong et al. (2012) proposed a facile electrochemical approach for synthesis of zirconia–reduced graphene oxide nanosheets for application in enzyme less methyl parathion sensor. Efforts have also been made to detect hydroquinone and catechol using RGO–$ZrO_2$ based sensor (Vilian et al., 2014).

We report for the first time results of the studies relating to application of zirconia decorated reduced graphene oxide nanocomposite as a transducer for development of an immunosensor. This immunosensor can be used for non-invasive, label-free and efficient detection of oral cancer biomarker (CYFRA-21-1) and it covers the entire physiological range (3.8 to$17.46 \pm 1.46$ ng $mL^{-1}$) secreted in saliva of oral cancer patients. The results obtained have been validated via enzyme linked immunosorbent assay (ELISA).

## 2. Materials and methods

### 2.1. Chemicals

Zirconium ethoxide, natural graphite flakes and 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich. Sodium hydroxide, cetyltrimethylammonium bromide (CTAB), sodium monophosphte, sodium diphosphate dihydrate and N-hydroxysuccinimide (NHS) were purchased from Fisher Scientific. 3-aminopropyl triethoxy saline (APTES) was purchased from Alfa-aesar. All the chemicals were of analytical grade and used without any further purification. Electrochemical studies were performed using 0.05 M phosphate buffer solution (PBS) prepared using sodium monophosphte and sodium diphosphatedihydrate. All solutions prepared using milli-Q water having resistivity of 18 MΩ cm were stored at 4 °C. CYFRA-21-1 antigen and anti-CYFRA-21-1 antibodies were purchased from Ray Biotech, Inc., India. These biomolecules were further diluted by using PBS buffer of pH 7.0. CYFRA-21-1 ELISA Kit was purchased from Kinesis DX, USA.

### 2.2. Fabrication of biosensing platform

Nanostructured $ZrO_2$ decorated RGO was prepared and functionalized with APTES (please see Supporting Information). 20 mg of functionalized nanocomposite (APTES/$ZrO_2$–RGO) was dispersed in 50 mL of acetonitrile by mild ultrasonication. The electrophoretic deposition (EPD) technique was used to deposit thin film of APTES/$ZrO_2$–RGO onto prehydrolyzed ITO glass that worked as an anode and platinum wire as cathode, placed parallely at a separation distance of 1 cm in glass cell. These were dipped in the prepared colloidal solution and electrophoretically deposited at an optimized DC potential (15 V) for about 3 min using electrophoretic deposition unit (Genetix, GX300C). This APTES/$ZrO_2$-RGO/ITO film was washed with deionized water and dried at room temperature (25 °C) over night. Next, a stock solution (50 μg $mL^{-1}$) of anti-CYFRA-21-1 was prepared in PBS (pH = 7.0). For activation of the –COOH groups of antibodies, EDC–NHS chemistry was used where EDC (0.2 M) worked as a coupling agent and NHS (0.05 M) as an activator. A solution mixture of anti-CYFRA-21-1, EDC and NHS in 2:1:1 ratio was made and kept at room temperature (25 °C) for 30 min. Further, 30 μL of activated antibody solution was uniformly spread over APTES/$ZrO_2$–RGO/ITO electrode and kept in a humid chamber at room temperature (25 °C) during the next 3 h. Thereafter, it was washed with PBS to remove any unbound antibody molecules. 20 μL of BSA (2 mg $dL^{-1}$) was used for blocking the non-specific active sites. The fabricated BSA/anti-CYFRA-21-1/APTES/$ZrO_2$–RGO/ITO immunoelectrode was washed with PBS and stored at 4 °C until further use.

### 2.3. Sample collection and processing

Saliva samples of eight OC patients were collected from Rajiv Gandhi Cancer Institute and Research Centre (RGCIRC), Delhi (India) after written consent by the patients. Prior to this,we took ethical approval of the Institutional Review Board of RGCIRC and Institutional Ethical and Biosafety Committee, DTU. We collected un-stimulated saliva samples after rinsing the mouth with 5 mL deionized water and thereafter expectorated into sterilized tube. These samples were centrifuged at 2800 rcf (25 °C) for 30 min. the supernatant was collected in sterilized eppendorf and stored at −20 °C until further use (Rajkumar et al., 2015). All the samples were collected, processed and stored in a similar fashion.

## 3. Results and discussion

### 3.1. X-ray diffraction (XRD) and Electron microscopy studies

Fig. 1a shows the XRD pattern obtained for the hydrothermally synthesized $ZrO_2$–RGO nanocomposite. The peak observed at 24.2° is due to merging of the $ZrO_2$ (110) and RGO (002) planes. Further, the relatively lower intensity broad peak is observed at 43.0°