Sensors and Actuators B 190 (2014) 723–729

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Epitaxial graphene immunosensor for human chorionic gonadotropin

Sofia Teixeira[a,*], G. Burwell[a], A. Castaing[a], D. Gonzalez[b], R.S. Conlan[b], O.J. Guy[a]

[a] College of Engineering, Swansea University, Singleton Park, Swansea, UK
[b] College of Medicine, Swansea University, Singleton Park, Swansea, UK

ARTICLE INFO

Article history:
Received 24 June 2013
Received in revised form 28 August 2013
Accepted 4 September 2013
Available online 23 September 2013

Keywords:
Epitaxial graphene
Immunosensor
Human chorionic gonadotropin
SiC
Amperometric
APTES

ABSTRACT

Human chorionic gonadotropin (hCG), a 37 kDa glycoprotein hormone, is a key diagnostic marker of pregnancy and has been cited as an important biomarker in relation to cancerous tumors found in the prostate, ovaries and bladder.

A novel chemically-modified epitaxial graphene diagnostic sensor has been developed for ultrasensitive detection of the biomarker hCG. Multi-layer epitaxial graphene (MEG), grown on silicon carbide substrates, was patterned using electron beam lithography to produce channel based devices. The MEG channels have been amine terminated using 3-Aminopropyl-triethoxysilane (APTES) in order to attach the anti-hCG antibody to the channel.

Detection of binding of hCG with its graphene-bound antibody was monitored by measuring reduction of the channel current of the graphene biosensor. The sensitivity of the sensor device was investigated using varying concentrations of hCG, with changes in the channel resistance of the sensor observed upon exposure to hCG. The detection limit of the sensor was 0.62 ng/mL and the sensor showed a linear response to hCG in the range 0.62–5.62 ng/mL with a response of 142 Ω/ng/mL. At concentrations above 5.62 ng/mL the sensor begins to saturate.

1. Introduction

Electrochemical immunosensors offer a number of significant advantages, including high sensitivity, fast response, simplicity, and relatively low cost [1,2] when compared with other types of immunosensor. These advantages make them attractive for high performance analytical detection of biomolecules [3]. Immunosensors capable of detecting nucleic acids [4], viruses [5], antigens [6], and hormone [7] biomarkers, based on silicon nanowire [5], carbon nanotube (CNT) [8], graphite [9] have been widely reported. Since the pioneering work on SiNW sensors by Lieber et al. [10], SiNW sensors have been developed that are capable of detection limits down to fg ml^{-1} concentrations–such as reported for prostate specific antigen (PSA) detection by Kim et al. [11]. CNT sensors have also been used to achieve detection of antigens e.g. PSA at ng/mL [8,12].

More recently, graphene has been utilized in a number of forms for sensor and biosensor applications [13–17].

Graphene's electronic structure and high surface to volume ratio contribute to the high sensitivity of graphene sensor devices [18]. Various forms of graphene and related materials (such as exfoliated graphene [19], graphene oxide and epitaxial graphene [20]), have been reported for use in graphene-modified electrodes and graphene-composite electrodes. An electrochemical sensor, using chemically modified exfoliated graphene to detect dopamine (DA), was reported to have a detection limit of 0.01 μM. [21]. Li et al. reported a novel electrochemical immunosensor of the breast cancer marker protein CA 15–3 using a highly conductive graphene-modified electrode. This sensor was capable of sensitive and label-free detection with a detection limit of 0.012 IU/mL [19]. Yasuhide et al. reported a label-free immunosensor based on an aptamer-modified graphene field-effect transistor (G-FET). The aptamer-modified G-FET showed selective electrical detection of IgE protein with an dissociation constant of 47 nM, indicating good affinity and the potential for G-FETs to be used in biological sensors [15]. Srivastave et al. reported an easy method for producing functionalized multilayer graphene from multiwalled carbon nanotubes (MWCNTs) in mass scale using only concentrated H_2SO_4/HNO_3. This biosensor shows linearity of 10–100 mg dL^{-1}, sensitivity of 5.43 μA mg^{-1} dL cm^{-2}, lower detection limit of 3.9 mg dL^{-1}, [22]. Lu et al. reported an hydrogen peroxide biosensor formed from single-layer graphene with a detection limit of 1.05×10^{-7} M [23]. Schedin et al. reported a sensor for detection of individual gas molecules adsorbed on graphene with a detection limit of 1 ppb [24].

Epitaxial growth on silicon carbide (SiC) is a widely used method of producing high quality graphene. Using insulating or semi-insulating SiC substrates enables the lithographic fabrication of graphene devices, for electronic applications without the need for

* Corresponding author at: College of Engineering, Swansea University, Singleton Park, SA2 8PP Swansea, UK. Tel.: +44 01792602816.
E-mail address: 607010@swansea.ac.uk (S. Teixeira).

0925-4005/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.snb.2013.09.019

transfer to other substrates [25,26]. Graphene can be grown on both the silicon and carbon faces of 4H-SiC, but growth is substantially different on each face, in terms of the morphology and electronic structure of the resulting graphene layers [27]. Silicon face growth yields an interfacial layer between the SiC substrate and graphene, and typically grows layer-by-layer [28]. In contrast, carbon face growth yields layers which are decoupled from the SiC substrate, without a noticeable interface effect [29]. Larger growth domains are produced on the carbon face, which contributes to higher carrier mobilities in a microchannel device [30].

Graphene biosensors can be fabricated by covalent immobilization, of proteins to graphene surfaces [31,32]. Covalent immobilization of antibody molecules onto graphene requires a chemical reaction of the COOH functional groups of the antibody, with amine groups, bound to the graphene surface, producing a peptide bond. Methods for amine functionalization of graphene include diazotization [33], thiol chemistry [34] and surface silanization using APTES. APTES attachment can be performed on various substrates provided they contain surface hydroxyl groups, which can react with alkoxysilanes to form covalent Si—O—C bonds to the underlying substrate.

An epitaxial graphene immunosensor, capable of selectively detecting hCG, has been developed. Human chorionic gonadotropin (hCG) is a glycoprotein hormone composed of 244 amino acids with a molecular mass of 36.7 kDa, produced by normal trophoblast cells of the placenta during pregnancy [35]. Trophoblast cells form the outer layer of a blastocyst, which provide nutrients to the embryo and develop into a large part of the placenta. Trophoblasts are formed during the first stage of pregnancy and are the first cells to differentiate from the fertilized egg. hCG is also produced by trophoblast cells in hydatidiform moles and choriocarcinoma (trophoblast diseases) in patients with germ cell tumors (testicular choriocarcinoma, placental site tumors and germ cell carcinomas of the ovary) and sometimes in patients with other malignancies [35].

In this work, standard lithographic techniques have been used to pattern epitaxial graphene devices, whose dimensions are scalable and suitable for wafer scale production. Epitaxial graphene devices have been functionalized using APTES, the first reported use of APTES on epitaxial graphene devices, to achieve an amine-terminated graphene surface. Antibodies targeted against hCG have been bound to the amine-terminated graphene in the first reported amperometric immunosensor based on epitaxial graphene. Device fabrication and surface functionalization methods are readily adaptable to other antibody/antigen systems, and are thus suitable as a generic immunosensor platform.

2. Experimental

2.1. Materials and reagents

Semi-insulating 4H-SiC substrates (nominally on-axis) were purchased from CREE. Synthetic urine was prepared with urea, sodium chloride, potassium chloride and sodium phosphate purchased from Sigma–Aldrich. *N*-Hydroxysuccinimide (NHS) and *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDAC), di-tert-butyl dicarbonate (t-BOC), sodium bicarbonate, bovine serum albumin (BSA), phosphate buffered saline (PBS), Potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate II ($K_4[Fe(CN)_6]$) trihydrate, and trifluoroacetic acid were all purchased from Sigma–Aldrich. Electrochemical measurements of the surface modification were performed using an aqueous reference electrode (Ag/AgCl) purchased from IJ Cambria Scientific Ltd. and platinum (Pt) auxiliary electrode from BASi. hCG protein was purchased from Abcam (UK). Anti-hCG antibody was supplied by Ig Innovations.

2.2. Apparatus

Graphene growth was performed in a Jipelec rapid thermal processing SiC furnace fitted with a turbo molecular pump. An e-Line (Raith GmBH) electron beam lithography system was used to define the graphene channels and contacts of the sensor device. Plasma etching of the graphene device was performed in an Oxford Instruments PlasmaLab. Cyclic voltammetry measurements were performed using an EmStat^2 Palm Sens potentiostat with the graphene channel device as the working electrode, a Pt auxillary electrode and Ag/AgCl reference electrode. Electrical measurements were performed using a Semi Probe LA-150 probe station with a Keithley 2602A Source Meter. Micro-Raman measurements were performed using a Renishaw InVia system with a 100 mW 532 nm excitation laser with approximately 10 mW of power on the sample. X-ray photoelectron spectroscopy (XPS) measurements were performed in a VG ESCAlab MKII with an Al X-ray excitation source (Kα of 1486 eV).

2.3. Graphene growth and device fabrication

4H-SiC wafers were cut into 10×10 mm samples and cleaned using a standard RCA procedure. Samples were etched in HF immediately prior to furnace growth to remove the native oxide. Multilayer graphene (MLG) was grown on the C-face of 4H-SiC at 1650 °C and a vacuum of 10^{-4} mbar.

Electron beam lithography (EBL) followed by oxygen plasma etching (50 sccm O_2, 75 mTorr and 50 W RF power, 60 s) was used to pattern graphene channel devices. A second EBL exposure was used to define the sputtered Ti/Au metal contacts to the graphene device. A third EBL exposure was used to define a window in PMMA, thus exposing only the graphene channel and protecting the metal contacts and the SiC surface from any chemical exposure during surface modification steps.

2.4. Hydroxylation of surface

The surface of the graphene channel devices was modified using the Fenton reaction [36], yielding an –OH terminated graphene layer. Epitaxial graphene samples were immersed in a solution of hydrogen peroxide and iron (II) sulfate, maintained at pH 3 for 30 min. Since the reaction is strongly exothermic, the iron sulfate powder was added to the solution incrementally and allowed to settle before introducing the graphene sample.

Contact angle measurements taken before and after the –OH termination reaction showed that the graphene surface changed from hydrophobic (90°) to hydrophilic (26°) following –OH termination.

2.5. Electrochemical assays

Cyclic Voltammetry (CV) measurements were conducted in 5.0 mmol/L of $[Fe(CN)6]^{3-}$ and 5.0 mmol/L of $[Fe(CN)6]^{4-}$, prepared in PBS buffer, pH 7.4. For CV assays, the potential was scanned from −0.7 V to +0.7 V, at 50 mV/s. All assays were conducted in triplicate.

2.6. Antibody immobilization with APTES

The –OH terminated graphene surfaces were reacted in a solution of 40% (3-Aminopropyl) triethoxysilane (APTES) in ethanol for 1.5 h, to obtain an amine-terminated surface (Fig. 1). Amine groups of the anti-hCG antibody were protected using di-tert-butyl dicarbonate (t-BOC) to prevent cross-linking and aggregation of antibodies. This also ensures that only the amine-terminated surface of the graphene channel binds to the carboxylic group of the antibody.