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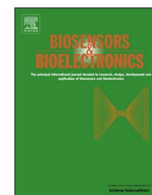

Multi-nanomaterial electrochemical biosensor based on label-free graphene for detecting cancer biomarkers

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ABSTRACT

Developing a rapid, accurate and sensitive electrochemical biosensor for detecting cancer biomarkers is important for early detection and diagnosis. This work reports an electrochemical biosensor based on a graphene (GR) platform which is made by CVD, combined with magnetic beads (MBs) and enzyme-labeled antibody-gold nanoparticle bioconjugate. MBs coated with capture antibodies (Ab1) were attached to GR sheets by an external magnetic field, to avoid reducing the conductivity of graphene. Sensitivity was also enhanced by modifying the gold nanoparticles (AuNPs) with horseradish peroxidase (HRP) and the detection antibody (Ab2), to form the conjugate Ab2–AuNPs–HRP. Electron transport between the electrode and analyte target was accelerated by the multi-nanomaterial, and the limit of detection (LOD) for carcinoembryonic antigen (CEA) reached 5 ng mL^{-1}. The multi-nanomaterial electrode GR/MBs–Ab1/CEA/Ab2–AuNPs–HRP can be used to detect biomolecules such as CEA. The EC biosensor is sensitive and specific, and has potential in the detection of disease markers.
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1. Introduction

A biomarker is a characteristic that is objectively measured and evaluated, as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention (Atkinson et al., 2001). Evaluating serum protein biomarker levels complements methods of detecting and staging tumors, tracking tumor recurrence or metastasis, determining responses to therapies, and estimating the prognosis of such patients. Developing highly sensitive biosensors to detect biomolecules such as cancer protein biomarkers (Chikkaveeraiah et al., 2012) has potential in the early detection of cancer.

Various strategies have been developed for detecting cancer biomarker proteins, including enzyme-linked immunosorbent assay (ELISA) (Voller et al., 1978), radioimmunoassay (RIA) (Goldsmith and Stanley, 1975), electrophoretic immunoassay (Schmalzing and Nashabeh, 1997) and mass spectrometry-based proteomics (Aebersold and Mann, 2003). These techniques often involve sophisticated instrumentation, significant sample volumes, limited sensitivity, and clinically unrealistic expense and measurement times. A simple, rapid, sensitive and economical method for protein measurement at point-of-care and in research applications is required.

Electrochemical (EC) sensors exhibit high sensitivity and specificity, and involve relatively simple instrumentation. They can potentially be expanded into multiplex detection platforms (Liao et al., 2006; Mani et al., 2009; Wei et al., 2008). Voltammetry (linear sweep, differential pulse, square-wave and stripping) and amperometry are the most widely used EC detection methods. They involve a tracer antibody labeled with an electroactive species, such as an enzyme, metal nanoparticle or quantum dot. The tracer is allowed to bind with an analyte, possibly through an intermediate primary antibody, and is thus immobilized on an electrode surface. The concentration of the targeted biomarker is quantified by applying a potential, and measuring the resulting current at the electrode. A variety of immunosensors for detecting cancer biomarkers are available.

The recent development of nanotechnology has advanced EC sensor detections. Nanomaterials can be used in various aspects of the detection system, including in capture probes, reporting molecules and electrode fabrication and coatings (Lord and Kelley, 2009; Ozsoz et al., 2003; Pandey et al., 2008; Radwan and Azzazy, 2009;

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Zhu et al., 2012). Much attention has been focused on signal amplification using nanomaterial as carriers in EC sensors.

Graphene (GR) is an excellent nanomaterial for EC applications, with its two-dimensional sheets of sp^2-hybridized C atoms in chexagonal configuration (Pumera et al., 2010). The properties of GR include fast electron transportation, high thermal conductivity, excellent mechanical stiffness and good biocompatibility (Li et al., 2008), which result in its promising application in nanocomposites (Stankovich et al. 2006), field-effect transistors (Lee. et al., 2012; Lin. et al., 2013), electromechanical resonators (Bunch et al., 2007) and solar cells (Hsu et al., 2012). The reduced graphene nanowalls (RGNWs) were efficient in label-free detection of single nucleotide polymorphisms of 20 zM oligonucleotides (~10 DNA/mL) with a specific sequence and could effectively contribute to the development of ultra-high-sensitive electrochemical biosensors with single-DNA solution (Akhavan et al., 2012).

Gold nanoparticle-based probes exhibit high sensitivity and stability and low cost, which are advantageous in rapid high-throughput detection methods. Gold nanoparticles (AuNPs) have high surface areas and interesting physicochemical properties. They can be conjugated with DNA, antibodies, enzymes and other biomolecules (Jia et al., 2009). AuNPs are used widely in developing biochemical detection platforms, for detecting various substances including algal cells and toxins (Gas et al., 2010; Zhou et al., 2009), insecticides (Blažková et al., 2009; Wang et al., 2005), metal ions (Tang et al., 2010; Zhou et al., 2010) and proteins (Dykman et al., 2005; Harrison et al., 2008; Sánchez-Martínez et al., 2009). Nanoscale structures of magnetic beads (MBs) have fast reaction kinetics compared with bulk solid surfaces, high surface area per unit volume (owing to their small diameter) and good stability. The relative ease of MBs′ surface modification with functional groups, DNA, enzymes or antibodies has led to their use in the development of sensitive, rapid EC immunoassay systems (Mani et al., 2011; Xie and Jon, 2012).

An EC sensor based on multi-nanomaterials was designed to be used as a potential alternative to detect the model protein carcinoembryonic antigen (CEA), with rapid response time and recovery time compared with the traditional detection, and also as a model protein detection with high sensitivity, specificity and stability. Patients with elevated pretreatment serum levels of CEA (Suzuki et al., 1999; Muley et al., 2003; Kulpa et al., 2002; Nisman et al., 1999; Sawabata et al., 2002) have shorter survival times than those with normal marker concentrations (CEA < 10 ng mL^{-1}). GR, MBs and AuNPs were used to amplify the detection signal. MBs coated with capture antibodies (Ab1) were attracted to isolated GR sheets by an external magnetic field, to give MBs–Ab1. This avoided reducing the conductivity of GR. Sensitivity was also enhanced by modifying the gold nanoparticles (AuNPs) with horseradish peroxidase (HRP) and the detection antibody (Ab2). This resulted in a CEA detection limit of 5 ng mL^{-1}, meeting the requirements for clinical diagnosis (Molina et al., 2003).

2. Materials and methods

2.1. Chemicals and materials

HRP, BSA, 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide-hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) were obtained from Sigma-Aldrich (CA, USA), and ammonium persulfate ($(NH_4)S_2O_8$) and acetone from Ling Feng Chemical Reagent Co. Ltd. (Shanghai, PR China). Potassium hexacyanoferrate were obtained from Sinopharm Group Co. Ltd. (Beijing, PR China). Carboxylic magnetic beads (diameter ~1 um, composed of highly cross-linked with magnetic material precipitated in pores evenly distributed throughout the beads and further coated with a carboxyl layer of that seals the iron inside the beads) were purchased from Invitrogen (CA, USA). CEA was purchased from Medix Biochemica (Finland). 0.01 M phosphate-buffered saline (PBS, pH 7.4) and 50 mM 2-(N-morpholino) ethanesulfonic acid (MES, pH 6.0) were used as the incubating and washing buffers.

2.2. Preparation of graphene electrode

Graphene films were primarily grown on 25 μm thick Cu through chemical vapor deposition (CVD). The growth process is as follow: (1) the fused silica tube was loaded with the Cu soil, evacuated, backed fill with hydrogen, heated to 1000 °C and maintained a H_2 pressure of 40 mTorr under a 2 sccm flow; (2) the Cu film was stabilized at the desired temperatures, up to 1000 °C,and introduced 35 sccm of CH_4 for a desired period of time at a total pressure of 500 mTorr; and (3) after exposure to CH_4, the furnace was cooled to room temperature. Then the copper substrate was dissolved by $(NH_4)S_2O_8$ solution within 4 h. The concentration of $(NH_4)S_2O_8$ solution is 30–50 g/L. The graphene was rinsed for 5 times with ultrapure water after etching. In this process glass sheet was used to refloat the graphene thin layer. The cleaned graphene was fixed on the Si+SiO_2 substrate, then put in the drying oven at 80 °C for 30 min. The substrate with graphene was dipped in acetone for 12 h to remove the organic polymer material PDDA. Finally, the graphene was treated by thermal annealing after removing the PDDA.

2.3. Preparation of Ab2–AuNPs–HRP

20 nm diameter colloidal AuNP solutions were prepared as described by (Zhou et al. 2012). Ab2–AuNPs–HRP was prepared as follows. A 200 μL solution of colloidal AuNPs was adjusted to pH 6.8 using 2 μL of K_2CO_3 solution (0.1 wt%). A 2 mL aqueous solution containing 80 μM EDC and 80 μM NHS was added, to activate the surface of the nanoparticles. Ab2 (1 μL, 1 mg mL^{-1}) and HRP (1 μL, 1 mg mL^{-1}) were added. Biomolecules were electrostatically adsorbed to negatively charged nanoparticle surfaces. After 10 min, the solution was centrifuged at 4 °C and 9000 rpm for 50 min. The supernatant was discarded, and the solids resuspended in 100 μL of PBS containing BSA (0.1 wt%).Ab2–AuNPs–HRP was stored at 4 °C until use.

2.4. Preparation of MBs–Ab1

MBs (50 μL, 10 mg mL^{-1}) were washed for 10 min with MES (50 μL, pH 6.0) twice. The vessel containing the solution was placed on a magnet, and the resulting supernatant discarded. An aqueous solution of EDC (50 μL, 50 mg mL^{-1}) and NHS (50 μL, 5 mg mL^{-1}) in MES was added to the washed MBs, and the resulting solution incubated in a hybridization instrument for 30 min at 25 °C and 4 rpm. The supernatant was again discarded under magnetization conditions. Activated MBs were washed with PBS twice, and the supernatant was removed by magnetization. 1 mg of MBs were added to 50 μg of Ab1 (1 μg mL^{-1}), and incubated in the hybridization instrument for 1 h at 25 °C and 4 rpm. Modified MBs were collected by magnetization, and tris–HCl (150 μL, 50 mM) in PBS was added to deactivate any unbound carboxyl groups. The coated MBs were washed with BSA (0.1 wt%) and Tween20 (0.1 wt%) in PBS, and the final MBs–Ab1 solution was stored at 4 °C until use.

2.5. Preparation of the multi-nanomaterial electrode

10 μL of target antigen was added to the MBs–Ab1 solution, and the resulting solution incubated at room temperature for 15 min. After discarding the supernatant by magnetization, 20 μL

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