Highly sensitive impedimetric immunosensor based on single-walled carbon nanohorns as labels and bienzyme biocatalyzed precipitation as enhancer for cancer biomarker detection

Fan Yang, Jing Han, Ying Zhuo, Zhehan Yang, Yaqin Chai [*], Ruo Yuan [*]

Education Ministry Key Laboratory on Luminescence and Real-Time Analysis, College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, People's Republic of China

ABSTRACT

A novel sandwich-type electrochemical immunosensor based on functionalized nanomaterial labels and bienzyme (horseradish peroxidase and glucose oxidase) biocatalyzed precipitation was developed for the detection of α-fetoprotein (AFP). The enzymes linked to functionalized nanomaterials as biocatalysts could accelerate the oxidation of 4-chloro-1-naphthol (4-CN) by H_2O_2 to yield the insoluble product on the electrode surface; the mass loading of the precipitates on the device led to a significant enhanced signal. Cyclic voltammetry and electrochemical impedance spectroscopy techniques were used to monitor the enhanced precipitation of 4-CN that accumulated on the electrode surface and subsequent decrement in the electrode surface area by monitoring the reduction process of the $Fe(CN)_6^{4-/3-}$ redox couple. Under optimal conditions, the proposed immunosensor showed a high sensitivity and a wide linear range from 0.001 to 60 ng mL^{-1} with a low detection limit of 0.33 pg mL^{-1}. Moreover, the immunosensor exhibited good selectivity, acceptable stability and reproducibility. The amplification strategy showed good promise for clinical screening of tumor biomarkers.

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1. Introduction

Cancer is currently one of the most common and threatening disease for human beings. In clinical analysis, there are some momentous associations between increased levels of tumor markers in human serum and patients with certain cancers. Thus, the sensitive and precise detection of tumor biomarkers plays an important role in early detection, monitoring disease recurrence, and therapeutic treatment efficacy to improve long term survival of cancer patients (Kulasingam and Diamandis, 1998). Due to the highly sensitive and selective nature of recognition between antigen and antibody, immunoassay has been considered as a major analytical technique for the quantitative determination of tumor markers. In the past decades, many immunoassay methods have been reported, such as optical (luminescence, fluorescence, surface plasmon resonance, etc.), electrochemical (amperometric, potentiometric, capacitive or impedance, etc.), and microgravimetric (quartz crystal microbalance, etc.) immunoassays (Ghindilis et al., 1998; Emon et al., 1998). Among these methods, electrochemical immunoassays received particular attention because of their simple

instrumentation, good portability, high sensitivity and fast analysis, and have been developed and extensively applied to the detection of tumor markers (Das et al., 2006).

Electrochemical impedance spectroscopy (EIS) is a powerful, nondestructive and informative technique for characterization of electrical properties of biological interfaces (Radi et al., 2009), which has received considerable attention as an electrochemical signal transducer in biosensors in recent years. It is well known that electrochemical impedimetric immunosensors, which are based on measuring the charge transfer resistance of a redox probe at an electrode interface, have been widely used to investigate the antibody–antigen interactions (Prodromidis, 2010). Elshafey et al. described development of a new sensitive label free electrochemical impedimetric immunosensor for the detection of Murine double minute 2 based on cysteamine self assembled monolayers on a clean polycrystalline Au electrode surface (Elshafey et al., 2013). Rezaei et al. developed an electrochemical impedimetric immunosensor for ultrasensitive determination of insulin-like growth factor-1 (IGF-1) based on immobilization of a specific monoclonal antibody on gold nanoparticles (GNPs) modified gold electrode (Rezaei et al., 2011). The designed impedimetric immunosensors have showed extensive linear and low detection limits. Despite many advances in this field, it is still a challenge to find new approaches that could improve the simplicity and sensitivity of electrochemical impedimetric immunosensor.

* Corresponding authors. Tel.: +86 23 6825 2277; fax: +86 23 6825 3172.
E-mail addresses: yaqinchai@swu.edu.cn (Y. Chai),
yuanruo@swu.edu.cn (R. Yuan).

Signal amplification is deemed as a vital point for sensitive immunosensors. Achieving high sensitivity has long been a major goal in immunoassay, especially in the monitoring of tumor markers. So far, there are various reports focusing on novel signal amplification, including nanomaterial labels, catalytically deposited nanoparticles, metal nanocarriers, multiple enzyme particles, dual amplification, catalytic chemical process and so on (Akter et al., 2012). Recently, much attention has been focused on signal amplification using nanomaterial labels to improve the signal amplification in the sandwich-type immunoassays (Pei et al., 2013). The growing exploration of nanotechnology has resulted in the identification of many unique chemical, physical and mechanical properties of nanomaterials, such as good biocompatibility, high surface-to-volume ratio, and enhanced magnetic and electrical properties (Liu and Webster, 2007; Murray, 2008). The unique properties of nanomaterials offer excellent prospects for interfacing biological recognition events with electronic signal transduction (Wang, 2005). Therefore, biomolecules labeled with nanomaterials could retain their bioactivity and effectively increase the biomolecules loading towards a sandwich complex reaction event, which further result in an enhancement of detection sensitivity (Peng et al., 2012; Liu et al., 2007; Ding et al., 2010). Single-walled carbon nanohorns (SWCNHs), as a novel carbon nanomaterial, not only have advantages of conventional carbon nanomaterials but also possess excellent catalytic properties, high-purity and low toxicities, which could be explored as a replacement of nanotubes to be used for electrochemical and biosensing study (Iijima et al., 1999).

In addition, enzymatic reactions are able to form polymeric layers on the electrode surface and this strategy also can be used for signal amplification. Enzymatic biocatalytic precipitation (BCP), involving the formation of insoluble precipitates on the electrode surface, has been utilized for enhancing the detection limits via mass amplification in the analytical fields (Patolsky et al., 2003). Amplification through the enzymatic precipitation of a nonconductive insoluble product has also been used for the development of biosensors. Yu′s group developed an ultrasensitive piezoelectric immunosensor for the detection of aflatoxin B_1 with enhanced amplification using horseradish peroxidase (HRP) induced biocatalytic precipitation (Jin et al., 2009). Chen′s group described a highly sensitive photoelectrochemical immunoassay with enhanced amplification using HRP induced biocatalytic precipitation on a CdS quantum dot modified electrode (Zhao et al., 2012). In this particular strategy, insoluble precipitates were produced through the biocatalytic reaction of labeled HRP to form an insulating layer on an electrode surface, which completely blocks the electron transfer process of a redox probe (such as ferricyanide ion). The extent of electrode insulation is monitored by electrochemical impedance spectroscopy and cyclic voltammetry. More importantly, further signal amplification could be achieved by using nanomaterials to immobilize a large number of HRP molecules.

In this work, a novel amplification strategy combined with the merits of nanomaterial labels and enzymatic biocatalytic precipitation was developed for amplified detection of AFP. To the best of our knowledge, enzymatically catalyzed precipitates based on bienzyme (HRP and glucose oxidase (GOx)) application for sandwich-type immunoassay were first reported. At first, the primary antibodies (Ab_1) were initially dropped onto the gold nanoparticles/graphene nanosheet nanocomposites (Au–Gra) modified electrode for subsequent immunorecognition between Ab_1 and analyte antigen (Ag). Then, the secondary antibodies (Ab_2) and a bienzyme (HRP and GOx) were covalently conjugated onto the surface of functionalized SWCNHs as molecular tags. In the presence of 4-chloro-1-naphthol (4-CN) and glucose, the catalyzed oxidation of glucose produces gluconic acid and H_2O_2.

Subsequently, the generated H_2O_2 oxidized 4-CN to form an insoluble and insulating product of benzo-4-chlorhexidine on the electrode surface in the presence of HRP. The insoluble precipitates on the modified electrode surface as an electrical barrier could efficiently block the interfacial electron-transfer of the redox probe at the electrode in the solution, and therefore detection sensitivity would be further enhanced. The detailed preparation, characterization, and desirable performance characteristics of the developed amplified immunoassay are described in the following section.

2. Experimental

2.1. Chemicals and materials

AFP and anti-AFP were purchased from Biocell Co. (Zhengzhou, China). SWCNHs were purchased from Jiansin Scientific & Trading Co. (Beijing, China). Graphene oxide sheets (GO) were obtained from Pioneer Nanotechnology Co. (Nanjing, China). Gold chloride tetrahydrate, sodium citrate, glucose, Bovine serum albumin (BSA, 96–99%), HRP, GOD, 4-CN, L-ascorbic acid (AA), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimidehydrochloride (EDC) and N-hydroxy succinimide (NHS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The phosphate buffer saline solution (PBS) was prepared by mixing the solutions of KH_2PO_4, Na_2HPO_4 and KCl. All chemicals and solvent used were of analytical grade and were used as received without further purification. Double distilled water was used throughout all experiments.

2.2. Apparatus

Cyclic voltammetric (CV) measurements and electrochemical impedance spectroscopy (EIS) measurements were performed using a CHI 660C electrochemistry workstation (Shanghai CH Instruments Co., China). A three-compartment electrochemical cell contained a modified glassy carbon electrode (GCE, $\Phi = 4$ mm) as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as a reference electrode. All potentials were measured and reported versus the SCE. The pH measurements were made with a pH meter (MP 230, Mettler-Toledo Switzerland) and a digital ion analyzer (Model pHS-3C, Dazhong Instruments, Shanghai, China). The sizes of nanomaterials were estimated from transmission electron microscopy (TEM; H600, Hitachi Instrument, Japan).

2.3. Synthesis of Au–Gra nanocomposites

Au–Gra nanocomposites were prepared by the following steps according to the literature (Han et al., 2012) with slight modification: graphene oxide (GO) sheets were first dissolved by ultrasonication in water. Then 0.1 g AA was added to 10 mL of aqueous dispersion of GO (1 mg mL^{-1}) and stirred for 10 h at room temperature. Subsequently, 2 mL of 1% gold chloride solution was added to the above mixture and stirred for 8 h. Finally, after three cycles of centrifugation and washing, the products of Au–Gra were dispersed in 5 mL of double distilled water.

2.4. Preparation of SWCNHs, bienzyme and anti-AFP bioconjugates

SWCNHs–bienzyme–Ab_2 bioconjugates were prepared through the carbodiimide coupling. Firstly, the carboxylation of SWCNHs was carried out according to the literature (Deng et al., Chem. Commun., (2012), 48). SWCNHs were ultrasonicated in an aqueous solution of 98% H_2SO_4, 68% HNO_3 and double-distilled water (1:3:6 in volume ratio) at 40 °C for 6 h to generate carboxylated SWCNHs.