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A novel label-free microfluidic paper-based immunosensor for highly sensitive electrochemical detection of carcinoembryonic antigen

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ABSTRACT

In this work, a highly sensitive label-free paper-based electrochemical immunosensor employing screen-printed working electrode (SPWE) for detection of carcinoembryonic antigen (CEA) was fabricated. In order to raise the detection sensitivity and immobilize anti-CEA, amino functional graphene (NH_2-G)/thionine (Thi)/gold nanoparticles (AuNPs) nanocomposites were synthesized and coated on SPWE. The principle of the immunosensor determination was based on the fact that the decreased response currents of Thi were proportional to the concentrations of corresponding antigens due to the formation of antibody–antigen immunocomplex. Experimental results revealed that the immunoassay enabled the determination of standard CEA solutions with linear working ranges of 50 pg mL^{-1} to 500 ng mL^{-1}, the limit of detections for CEA is 10 pg mL^{-1} (S/N=3) and its corresponding correlation coefficients were 0.996. Furthermore, the proposed immunosensor could be used for the determination of clinical serum samples. A large number of clinical serum samples were detected and the relative errors between measured values and reference concentrations were calculated. Results showed that this novel paper-based electrochemical immunosensor could provide a new platform for low cost, sensitive, specific, and point-of-care diagnosis in cancer detection.

1. Introduction

Lung cancer, whose cure has not yet been achieved, is one of the highest morbidity and mortality of malignant tumors in the world, and there is still an increasing trend year by year (Lefkowitz et al., 2010; Lee et al., 2015; Wang et al., 2013). Striving for "early diagnosis, early detection and early treatment" are the main measures to reduce the death rate of lung cancer. At present, the diagnosis of lung cancer is mainly on the basis of imaging examination, however, as to the early detection, it does have some limited effectiveness (Grose et al., 2015). In clinical analysis, the elevated levels of tumor markers in blood serum are associated with patients with certain cancers (Liu et al., 2010). CEA as one of the most widely used tumor markers, could be used in the clinical diagnosis of colorectal, gastric, pancreatic and cervical carcinomas. Meanwhile, the concentrations of CEA in blood serums are related to the stage of tumor, the outcome of therapy and the prognosis, so it can be used as a marker to directly evaluate curative effects, recrudescence, and metastasis (Miao et al., 2014). Therefore, sensitive, and accurate assays for the determination of CEA with low concentration level in complex biological samples are necessary for effective early diagnosis and therapeutics of cancer (Wulfkuhle et al., 2003; Hawkridge and Muddiman, 2009; Ferrari, 2005).

A number of methods can be used to monitor CEA, such as radioimmunoassay, chemiluminescence assay, and so on (Wang et al., 2012; Liu et al., 2015). Although these methods are sensitive, they typically require large instruments, and this makes the assay process more complex and time consuming, which cramps their blossoms. Since its introduction more than 30 years ago, ELISA has probably become the most used technique for such tasks, but the requirements of relatively expensive test kits and bulky plate readers limit ELISA's usefulness for point of care testing (POCT) (Chikkaveeraiah et al., 2012; Ricci et al., 2012). By contrast, label-free electrochemical immunosensor, which will perfectly meet the demand of point-of-care diagnosis, so as to become one of the research hot spots (Wilson, 2005; Wang, 2006). Combining with

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nanotechnology, label-free electrochemical techniques used in this work does not need pre-preparation of the antibody, which allows the introduction of reliable, highly sensitive, fast, low-cost, low determination limit, and highly selective electrical nano-devices for the detection of cancer biomarkers and provides better diagnostic accuracy. (Ionescu et al., 2004; Campanella et al., 1999; Skladal, 1997).

Paper made of cellulose fibres is naturally hydrophilic and allows penetration of aqueous liquids within its fiber matrix. This property provides the foundation for using paper to fabricate microfluidic systems (Li et al., 2010) Combining the advantages of paper with microfluidic chip technology, microfluidic paper-based analytical device (μPAD) was firstly put forward by Harvard chemical biologist J. Whitesides in 2007 (Martinez el al., 2007). Compared with traditional commercialized electrodes which were expensive, sometimes inconvenient, the paper-based microfluidic devices (μPADs) had emerged as low-cost alternative for quantitative chemical measurement (Martinez et al., 2008; Li et al., 2015). It possesses the strengths of good biocompatibility, low background and high sensitivity (Fenton et al., 2009; Lu et al., 2010). Owning to the merits mentioned above, it provides not only a new platform for disease diagnostic and environment monitoring, but also a promising way for people in developing countries to monitor their health (Yan et al., 2014; Wang et al., 2013).

Generally, fully functional paper-based microfluidic devices consist of microfluidic channels and electrodes. It has been reported that the microfluidic channels of μPADs can be built by many techniques including wax printing, inkjet printing, photolithography, flexographic printing, plasma treatment, laser treatment and so on (Elsharkawy et al., 2014; Rosenfeld and Bercovici, 2014). Compared with other methods, the wax printing involves the fewest number of steps and is best suited for fabricating large numbers of paper-based analytical devices in a single batch. The advantages of the method are a simple fabrication process, rapid, inexpensive and environmental friendly (Cai et al., 2013; Li et al., 2014). Thus, it is a very suitable technique for fabricating the microfluidic channels. As for electrodes, screen-printing technique offers a rapid and cost reducing way to fabricate robust and solid electrodes. A wide variety of inks could be printed on different kinds of substrates through screen-printing (Xia et al., 2015).

With rapid advances in the field of paper-based microfluidics, research efforts are shifting focus to the creation of fully functional μPADs with superior analytical performance for practical uses, for which the introduction of biosensing nanomaterials to μPADs has shown remarkable potential (Li et al., 2015). Graphene has attracted exceptional interest since it was discovered in 2004. It is a two-dimensional nanocarbon material with extraordinary physical and chemical properties (Novoselov et al., 2004), such as excellent electronic mobility (Bolotin et al., 2008), high specific surface area (Stoller et al., 2008), superb thermal conductivity (Balandin et al., 2008), high mechanical strength (Lee et al., 2008; Huang et al., 2012) and high chemical stability (Li et al., 2008). Because of its unique properties, graphene has been one of the most promising materials in designing electrochemical immunosensors (Mao et al., 2012; Su et al., 2011). Metal nanoparticles have also received a great deal of attention because of their fascinating physical and chemical properties, which are different than the bulk forms of the same materials (Kleijn et al., 2014). Among so many metal nanoparticles, gold nanoparticles (AuNPs) for its great chemical stability, large specific surface area, strong adsorption ability, good electrical conductivity, good suitability, and good biocompatibility, are more desirable for decorating graphene (Lv et al., 2014).

In this work, a highly sensitive label-free electrochemical immunosensor for detection of CEA employing a screen-printed carbon working electrode (SPWE) modified with graphene nanocomposites was developed onto the microfluidic paper analytical device. For the sensitive determination of CEA, NH_2-G/Thi/AuNPs nanocomposites were synthesized and coated on the SPWE for the immobilization of anti-CEA. The principle of the immunosensor determination was based on the fact that the decreased response currents of Thi were proportional to the concentrations of corresponding antigens due to the formation of antibody–antigen immunocomplex. This novel immunosensor would provide a new platform for low cost, sensitive, specific, and point-of-care diagnosis to public health.

2. Experimental

2.1. Apparatus

Cyclic Voltammetry and Differential Pulse Voltammetry were performed on an Autolab PGSTAT302N electrochemical workstation (Autolab, Herisau, Switzerland). A Dell D4500 computer was used to collect electrochemical data. Water was purified through a Michem ultrapure water apparatus (Michem, Chengdu, China, resistivity >18 MΩ). Scanning electron microscopy (SEM) images were recorded using an S-3500 scanning electron microscope (S-3500, Hitachi, Japan). The wax was sprayed by a Xerox ColorQube 8570 digital wax printer (Xerox, Norwalk, USA). Other apparatus included optical microscope (BX51TRF, Olympus, Japan) and ultrasonic generator (KH2200E, Hechuang, China).

2.2. Reagents

Graphene oxide was purchased (purity >95 wt%) from Xianfeng nanomaterials company (Nanjing, China). The phosphate-buffered saline (PBS, 0.1 M Na_2HPO_4-NaH_2PO_4-KCl, pH 7.4) was prepared from a PBS tablet (Sigma, St.louis, MO, USA). Thionine acetate (Thi) was obtained from Alfa Aesar. Chitosan (CS, from crab shells, minimum 85% deacetylated) was purchased from Sigma-Aldrich Co., Ltd. (St.louis, MO, USA). Bovine serum albumin (BSA) was purchased from Beijing Chemical Reagents Company (Beijing, China). Carbon ink (ED581 ss) was purchased from Acheson and Ag|AgCl ink (CNC-01) was from Yingman nanotechnology. Whatman chromatography paper No. 1 (200.0 mm × 200.0 mm, pure cellulose paper) was purchased from GE Healthcare Worldwide (Pudong Shanghai, China) and used with further adjustment of size. All other chemicals were of analytical reagent grade and used without further purification. All experiments were carried out at ambient temperature. Clinical serum samples were available from Peking University Cancer Hospital & Institute.

2.3. Design and fabrication of microfluidic paper-based analytical device

The microfluidic paper-based analytical device was fabricated on two pieces of selectively patterned Whatman No.1 cellulose filter paper (10.5 mm × 35.0 mm) through four steps. In the first approach, the structure of the immunodevice was designed by Adobe Illustrator CS5 on computer. A rectangular sample tab and a rectangular auxiliary tab comprised the paper-based devices of which detailed shape and size were shown in Scheme 1. A circle connecting zone of 4 mm in diameter was designed on the sample tab. Corresponding to it, there is one connecting zone (diameter = 6 mm) on the auxiliary tab. Three electrodes were used during measurement, including the working electrode, the counter electrode and the reference electrode. The designed pattern of working electrodes was shown in Scheme 1(A) (c) and counter electrode and reference electrode was in Scheme 1(B) (c). Many factors, such as operation convenience of the immunosensor are fully taken into account of in the design process. In addition, all