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A paper-based microfluidic electrochemical immunodevice integrated with amplification-by-polymerization for the ultrasensitive multiplexed detection of cancer biomarkers

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ABSTRACT

A novel signal amplification strategy for ultrasensitive multiplexed detection of cancer biomarkers using a paper-based microfluidic electrochemical immunodevice is described. Specifically, a controlled radical polymerization reaction is triggered after the capture of target molecules on the immunodevice surface. Growth of long chain polymeric materials provides numerous sites for subsequent horseradish peroxidase (HRP) coupling, which in turn significantly enhances electrochemical signal output. The signal was further amplified through the use of graphene to modify the immunodevice surface to accelerate the electron transfer. Activators generated electron transfer for atom transfer radical polymerization (AGET ATRP) was used in this study for its high efficiency in polymer grafting and better tolerance toward oxygen in air. Glycidyl methacrylate (GMA) was examined to provide excess epoxy groups for HRP coupling. In the electrochemical immunodevice, eight carbon working electrodes, as well as their conductive pads, were screen-printed on a piece of square paper, and the same Ag/AgCl reference and carbon counter electrodes were shared with another piece of square paper via stacking. Using the HRP-*O*-phenylenediamine-H_2O_2 electrochemical detection system, four cancer biomarkers: carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), cancer antigen 125 (CA125), and carbohydrate antigen 153 (CA153) were detected. A limit of detection of 0.01, 0.01, 0.05 and 0.05 ng mL^{-1} was demonstrated, respectively. The results show that the proposed strategy offers great promises in providing a sensitive and cost-effective solution for biosensing applications.

1. Introduction

Multiplexed immunoassay of biomarkers has recently attracted considerable interest due to its advantages in early screening of diseases, evaluating the extent of diseases, and monitoring the response of diseases to therapy (Geissler et al., 2012). Whitesides and coworkers introduced a promising concept of using patterned paper substrate as microfluidic platform for multiplex analyte detection (Martinez et al., 2007, 2008). Microfluidic paper-based analytical devices (μPADs) have gained more and more attention and great interest during the recent years (Schilling et al., 2012; Martinez et al., 2010; Sia and Kricka, 2008a; Mentele et al., 2012; Wang et al., 2013). It is a promising technology for point-of-care testing (POCT), public health and environmental monitoring applications, in which highly sensitive methods and complex function must be combined with low-cost, rapid and simple fabrication and operation (Sia and Kricka, 2008b; Whitesides, 2011).

To date, many methods have been developed for qualitative and quantitative analyses of multiplex analytes on μPADs, such as colorimetric method (Olkkonen et al., 2010; Abe et al., 2008; Bruzewicz et al., 2008; Lu et al., 2009; Noh and Phillips, 2010; Cheng et al., 2010; Apilux et al., 2010), electrochemical method (Wang et al., 2012a), chemiluminescent method (Yu et al., 2011) and electrochemiluminescent method (Ge et al., 2012b). Some of the methods not only retain the features of simplicity, low cost, portability and disposability of paper-based analytical devices, but also provide new opportunities and directions in the development of precise and sensitive diagnostic devices. Nevertheless, the increasing demand for screening diseases at their early stage of development calls for ultrasensitive detection of biologically

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relevant molecules at an extremely low level of expression, which inevitably leads to intense research efforts toward exploring novel means to enhance detection sensitivity. Some successful strategies include the employment of new redox-active probes, the integration of enzyme-assisted signal amplification processes, and the incorporation of nanomaterials to increase the upload of signal tags, etc (Karra et al., 2013; Fu et al., 2013; Xu et al., 2013; Coll et al., 2013; Lei and Ju, 2012). The last approach is particularly effective by introducing multiple signal tags per binding event. Similar to nanoparticles, long chain polymeric materials with numerous chemically modifiable functional groups are capable of providing extra signal tags in the same fashion. Indeed, the use of polymer films to increase the loading of signal tags has been routinely employed (Wu et al., 2009, 2010, 2011).

In this report, we described coupling of polymerization-assisted signal amplification with paper-based microfluidic electrochemical immunodevice for multiplexed detection of cancer biomarkers, in an attempt to further enhance sensing sensitivity as well as to provide an interface compatible to existing commercial sensing techniques based on electrochemical readouts. Paper-based microfluidic electrochemical immunodevice (Scheme 1A) was prepared based on the photoresist-patterning technique and screen-printed paper-electrodes. AGET ATRP was used in polymerization for its tolerance to oxygen in the air. Glycidyl methacrylate (GMA) was used as the monomers to provide epoxy groups for immobilization of electrochemical tags (HRP). Four cancer biomarkers, namely, carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), cancer antigen 125 (CA125), and carbohydrate antigen 153 (CA153), were used as model analytes. These biomarkers were detected using the HRP-*O*-phenylenediamine-H_2O_2 electrochemical system under optimized conditions. This method showed the effectiveness of the proposed strategies of signal amplification for the ultrasensitive detection of cancer biomarkers.

2. Experimental section

2.1. Materials and reagents

Human CEA, AFP, CA125, and CA153 standard solutions, mouse monoclonal capture and signal CEA, AFP, CA125, and CA153 antibodies, and HRP-labeled signal CEA, AFP, CA125, and CA153 antibodies were purchased from Meridian Life Science Inc. (TN, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), ascorbic acid, 2,2′-bipyridyl (bpy), $CuCl_2$, *N*,*N*-dimethylformamide (DMF), Tween-20, HRP, glutaraldehyde (25% aqueous solution), chitosan, bovine serum albumin (BSA), *O*-phenylenediamine, and H_2O_2 were obtained from Sigma-Aldrich (MO, USA). Glycidyl methacrylate (GMA) was obtained from Alfa Aesar (Ward Hill, MA) and purified in house to remove the inhibitor (Lou and He, 2006; Qian and He, 2009a, 2009b). A negative photoresist SU-8 3010 and developer were purchased from MicroChem Corp. (Newton, MA, USA). Carbon ink (ELECTRODEDAGPF-407C) and silver/silver chloride ink (ELECTRODAG7019 (18DB19C)) were purchased from Acheson (Germany). Whatman chromatography paper #1 (200.0 mm × 200.0 mm, pure cellulose paper) was obtained from GE Healthcare Worldwide (Pudong Shanghai, China) and used with further adjustment of the paper size. All other chemicals were of analytical grade and were used as received. Twice-distilled water was used throughout the study.

2.2. Apparatus

Differential pulse voltammetric (DPV) measurements and electrochemical impedance spectroscopy (EIS) were performed with a CHI 660C electrochemical workstation (CH Instrument Co., Shanghai, China). Scanning electron microscopic (SEM) images were obtained using a JEOL JSM-5510 scanning electron microscope (Japan). The UV–vis absorption spectra were recorded with a UV-3600 UV–vis-near-infrared (NIR) spectrophotometer (Shimadzu, Japan).

2.3. Fabrication of paper-based microfluidic electrochemical immunodevice

The paper-based microfluidic electrochemical immunodevice consists of two layers of selectively patterned square filter paper of the same size (35.0 mm × 35.0 mm), as shown in Scheme 1A. The patterned mask of this paper-based microfluidic electrochemical immunodevice was designed in two dimensions using AutoCAD2012. Paper-A contains a central connecting zone (diameter = 7.0 mm) surrounded by eight working zones (diameter = 4.0 mm). Corresponding to paper-A, there is one circular connecting zone (diameter = 7.0 mm) in the center of paper-B. The fabrication procedure of this immunodevice is the following: Whatman chromatography paper #1 was impregnated with SU-8 3010 photoresist and then spin-coated at 2000 rpm for 30 s to spread the photoresist over the paper uniformly. The photoresist-coated paper was pre-baked on a hotplate at 95 °C for 10 min. The paper was tightly covered with a pre-designed film photomask and irradiated with a UV light (345 nm, 17 mW cm^{-2}) for 30 s. After post-baking at 95 °C for 1 min, the unpolymerized photoresist was chemically washed away by immersing the paper into acetone for 1 min and rinsing with acetone. Then, the paper was dried in a vacuum oven for 10 min and was ready to use. The region soaked by photoresist is impermeable to liquid, whereas the photoresist-eluted region remains hydrophilic.

Eight working electrode zones were screen-printed with carbon ink in a specific area on paper-A. Similarly, carbon ink and Ag/AgCl ink were screen-printed on a predesigned area of paper-B as the counter electrode and the reference electrode, respectively. Eight working electrodes share one pair of counter and reference electrodes after the two paper layers were stacked together.

2.4. Synthesis of initiator coupled signal antibody

The *N*-hydroxysuccinmidyl bromoisobutyrate (initiator) was prepared according to previous report (shown in Supporting information) (Lou et al., 2005). We synthesized four kinds of initiator-coupled signal antibody: initiator-coupled CEA signal antibody, initiator-coupled AFP signal antibody, initiator-coupled CA125 signal antibody and initiator-coupled CA153 signal antibody. The prepared NHS-coupled initiator (10 mg mL^{-1} in DMF, 10 μL) was added to a CEA, AFP, CA125 or CA153 signal antibody solution at 10 mg mL^{-1}, respectively, where the molar ratio of initiator to antibody was controlled at 8:1. The mixture was stirred overnight to allow the coupling reaction reaching completion and the excess NHS ester to hydrolyze. The concentration of the solution was determined by UV absorbance at 280 nm, and then diluted with PBS buffer (0.1 M, pH 7.4) to 1 mg mL^{-1}.

2.5. Preparation of paper-based microfluidic electrochemical immunodevice

Graphene oxide (GO) was synthesized from graphite through the modified Hummers method (Li and Wu, 2009; Liu et al., 2010). The as-synthesized graphite oxide was suspended in water and subjected to dialysis for one week to remove any residual salts. After drying at 50 °C overnight, the as-purified graphite oxide was exfoliated into GO by ultrasonicating a 0.05 wt% aqueous dispersion for 30 min. The unexfoliated graphite oxide was removed