PAPER-BASED MICROFLUIDIC ELECTROCHEMICAL IMMUNODEVICES INTEGRATED WITH NANOBIOPROBES ON GRAPHENE FILM FOR ULTRASENSITIVE DETECTION OF CANCER BIOMARKERS

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ABSTRACT

We report a novel signal amplification strategy integrated into a microfluidic paper-based electrochemical immunodevice to perform multiplexed measurement of cancer biomarkers. The signal amplification was achieved by using graphene to modify the immunodevice surface to accelerate electron transfer and silica nanoparticle as a tracing tag to label signal antibodies. Accurate, rapid, simple and inexpensive point-of-care electrochemical immunoassays were demonstrated with a photoresist-patterned microfluidic paper-based analytical device. Using the horseradish peroxidase (HRP)-O-phenylenediamine-H₂O₂ electrochemical detection system, the potential clinical applicability of this device was demonstrated through its ability to identify four candidate cancer biomarkers in serum samples from cancer patients.

KEYWORDS: Paper-Based Microfluidic Device, Electrochemical Detection, Immunoassay, Cancer Biomarkers

INTRODUCTION

The microfluidic paper-based analytical device (µPAD) is a class of microfluidic systems capable of performing truly disposable, inexpensive, and portably total analysis, especially in that the microchannels patterned on paper can be used for sample pretreatment and separation [1]. It combines the simplicity of paper strip tests and the complexity of the conventional lab-on-chip devices. Multiplexed immunoassay of biomarkers on the paper platforms has recently attracted considerable interest due to its advantages in evaluating the extent of diseases, and monitoring the response of diseases to therapy [2,3]. To date, the primary and scanty immunoassay for the qualitative analysis of multiplex analytes on µPADs is based on colorimetric method. However, colorimetric assay is not sufficiently sensitive or specific for accurate point-of-care use. In addition, the visual readout is usually limited to a yes/no answer, which is not adequate if the level of an analyte is very critical. Nevertheless, the increasing demand for screening diseases at their early stage of development calls for ultrasensitive detection of biologically relevant species at an extremely low level of expression, which inevitably leads to intense research efforts toward exploring signal amplification strategy to enhance detection sensitivity. The presented paper is a first study of combining a novel signal amplification strategy with µPAD for quantitative analysis of four different cancer biomarkers simultaneously, namely, alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), and carbohydrate antigen 153 (CA153).

![Figure 1: The fabrication process of the microfluidic paper-based electrochemical immunodevice. Electrodes were screen-printed on paper-A and paper-B respectively; then silver paint was added as the conductive pad. Paper-A and paper-B were cut into the same size (35.0 mm × 35.0 mm). The right panel shows the photograph of a final microfluidic paper-based electrochemical immunodevice ready for use. The chip size is comparable to a US quarter dollar coin.](image)

EXPERIMENTAL

The device consists of two layers of selectively patterned square filter paper of the same size (Figure 1). Paper-A contains a central connecting zone (diameter 7.0 mm) surrounded by eight working zones (diameter 4.0 mm). In the same position of paper-B, there is one circular connecting zone of the same size. Whatman chromatography paper was soaked
with SU-8 photoresist and patterned by UV photolithography. The unpolymerized photoresist in the paper was removed by rinsing with acetone. Then, the paper was dried in a vacuum oven and 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 paper-B as the counter electrode and the reference electrode, respectively. Finally, more silver paint was applied as the conductive pads to connect the paper chip to an electrochemical workstation. Eight working electrodes share one pair of counter and reference electrodes after the two paper layers were stacked together back-to-back with four pairs of magnet. The hydrophilic patterns around the electrodes on paper-A and paper-B constituted reservoirs of the electrochemical cells.

The monodispersed SiO$_2$ nanoparticles was synthesized by seed-growth methods [4]. We prepared four types of nanobioprobes corresponding four different cancer markers: HRP and antibody coimmobilized on SiO$_2$ nanoparticles. Figure 2A shows the process used for the preparation of the nanobioprobes, AFP as a model. Graphene oxide (GO) was synthesized from graphite using the modified Hummers method. The working electrodes were subsequently processed with GO solution, 0.05% chitosan solution, electrochemical reduction, and incubated with glutaraldehyde. Then, four types of capture antibodies were immobilized on the corresponding working electrodes that can capture the synthesized nanobioprobes through sandwiched immunoreactions. Figure 3 indicates the successful modification of the surface of the immunodevice after each step.

![Figure 2: (A) Preparation of nanobioprobes: coimmobilization of HRP and antibody on monodispersed SiO$_2$ nanoparticles; (B) fabrication and assay procedure, AFP as an example.](image)

The electrochemical assay procedures used with the prepared microfluidic paper-based electrochemical immunodevice are shown in Figure 2B. 2.0 µL of the sample solution containing different concentrations of cancer markers was added to each working electrode and incubated. Then, 2.0 µL of 10 mg mL$^{-1}$ HRP/Ab/SiO$_2$ was added to the corresponding working electrodes and incubated. For detection, 40 µL of 10.0 mM PBS buffer, containing 2.0 mM O-phenylenediamine and 4.0 mM H$_2$O$_2$ were added to the center of the paper electrochemical cell, and the eight working electrodes were sequentially placed into the electrical circuit to trigger the electrochemical reaction. In the presence of the HRP-labeled immunocomplexes on the paper, the electroactive species 2,2'-diaminoazobenzene was first produced. The Differential Pulse Voltammetric (DPV) measurements were then performed.

**RESULTS AND DISCUSSION**

The analytical performance of this method was verified by applying 2.0 µL of samples of human AFP, CEA, CA125, and CA153 standard solutions at various concentrations in PBS under the abovementioned optimized conditions. The DPV response and calibration curves for the four cancer biomarkers are shown in Figure 4. With increasing concentrations of AFP, CEA, CA125, and CA153, good correlations between the concentration of the cancer biomarkers and the DPV response were observed with similar wide dynamic ranges (0.001-100 ng mL$^{-1}$, 0.005-100 ng mL$^{-1}$, 0.001-100 ng mL$^{-1}$, and 0.005-100 ng mL$^{-1}$, respectively), which corresponded to the levels that occur naturally in human blood plasma or serum. The lowest detectable concentrations for the four cancer biomarkers were 0.001 ng mL$^{-1}$, 0.005 ng mL$^{-1}$, 0.001 ng mL$^{-1}$, and 0.005 ng mL$^{-1}$, respectively. These levels were much lower than those reported in earlier studies [5].
An ideal electrochemical immunoarray must exclude cross-talk resulting from the diffusion of the signal reporter from one electrode to the neighboring electrodes. In our strategy, the diffusion of the signal reporter, SiO$_2$ nanoparticles, was significantly hindered by the barrier structure of highly porous paper channels. As a consequence, any potential cross-talk should be reduced to a minimum. To further confirm the resistance to cross-talk, the cross-reactivity between the analytes and non-cognate antibodies was investigated. The cross-reactivity was evaluated by comparing the DPV responses obtained when the device was either incubated with a blank solution or 100 ng mL$^{-1}$ of AFP, CEA, CA125, or CA153. We found that only the working electrode prepared with the corresponding capture antibodies yielded obvious DPV responses (Figure 5). The cross-reactivity detected between the analytes and non-cognate antibodies was also negligible. Therefore, it is feasible to perform simultaneous multi-analyte immunoassays with this device.

CONCLUSION

This work is the first demonstration of the introduction of a signal amplification strategy into a simple microfluidic paper-based electrochemical immunodevice for the ultra-sensitive and high-throughput detection of cancer biomarkers in a multiplex point-of-care diagnostic format. The introduction of graphene onto the surface of the immunodevice efficiently accelerated the electron transfer and enhanced the detection signal. An additional signal amplification step was achieved by reducing the physical adsorption using silica nanoparticles as a label. As a consequence, a large amount of HRP could be introduced onto the SiO$_2$ nanoparticle carriers to maximize the ratio of enzyme per sandwich immunoassay. The immunodevice proposed in this work combines the simplicity and low cost of microfluidic paper-based immunodevices with the ultra-sensitivity of signal amplification strategies. The newly designed immunodevice was demonstrated to be very efficient and ultra-sensitive in the detection of low levels of cancer biomarkers.

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