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Paper-based chemiluminescence ELISA: Lab-on-paper based on chitosan modified paper device and wax-screen-printing

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ABSTRACT

A novel lab-on-paper device combining the simplicity and low-cost of microfluidic paper-based analytical devices (μPADs) and the sensitivity and selectivity of chemiluminescence ELISA (CL-ELISA) for the high-throughput, rapid, stable and reusable point-of-care testing is presented here. Chitosan was used to modify μPADs to covalently immobilize antibodies on μPADs. Thus, sandwich CL-ELISA on μPADs can be easily realized for further development of this technique in sensitive, specific and low-cost application. The paper device was fabricated by a low-cost, simple, and rapid wax-screen-printing method. Using tumor markers and paper microzone plate as model, the application test of this paper-based CL-ELISA was successfully performed with a linear range of 0.1–35.0 ng mL^{-1} for α-fetoprotein, 0.5–80.0 U mL^{-1} for cancer antigen 125 and 0.1–70.0 ng mL^{-1} for carcinoembryonic antigen. Since the cutoff values of the three tumor markers in clinical diagnosis are 25 ng mL^{-1}, 35 U mL^{-1} and 5 ng mL^{-1}, the sensitivity and linear ranges of the proposed method were enough for clinical application. In addition, this lab-on-paper immunodevice can provide reproducible results upon storage at 4 °C (sealed) for at least 5 weeks. Ultimately, this novel chitosan modification and wax-screen-printing methodology for μPADs can be readily translated to other signal reporting mechanism including electrochemiluminescence and photoelectrochemistry, and other receptors such as enzyme receptors and DNA receptors for determination of DNA, proteins and small molecules in point-of-care testing.

1. Introduction

In recent years, there has been a growing interest in improved point-of-care testing (POCT) diagnostics of analytes (including proteins, DNA/RNA) for home care, individualized medicine, and therapeutics (Yager et al., 2006; Cheng et al., 2009; Jokerst et al., 2008; Curtis et al., 2008). Paper cellulose fiber web with high surface area has been used extensively as a platform in analytical and clinical chemistry (Clegg, 1950) due to the fact that it is abundant, inexpensive, sustainable, disposable and easy to use, store, and transport and familiar to the public (Zhao et al., 2008; Martinez et al., 2007; Bruzewicz et al., 2008; Martinez et al., 2008a). Paper-based bioanalysis dates back to the early 20th century and the invention of paper chromatography in 1952, for which Martin and Synge were awarded the Nobel Prize in chemistry, is a big breakthrough. The most prevalent example is immunochromatographic tests (Lode, 2005; Mabey et al., 2004), such as diabetes and pregnancy tests strip. These conventional paper strip tests have gained great success in POCT diagnostics due to their simplicity, however they are normally not capable of doing multiplex and quantitative analysis (Lode, 2005; Lim et al., 2005), which is still needed when the level of an analyte in complex samples is very important.

These problems can be potentially improved by the recently developed microfluidic paper-based devices (μPADs) (Martinez et al., 2007, 2008a; Bruzewicz et al., 2008), which combine the mentioned advantages of paper tests strip with the complexity and multi-function of the conventional lab-on-chip devices. This promising technique has the potential to be good alternatives for POCT over traditional lab-on-chip devices. Another superiority for using paper as a microfluidic substrate is that high speed coating and printing techniques have been available for paper substrates, such as photolithography (Martinez et al., 2007, 2008a,b), plotting (Bruzewicz et al., 2008), plasma oxidation (Li et al., 2008), cutting (Fenton et al., 2009) and inkjet printing (Abe et al., 2008). Each method has its own set of advantages and limitations. Wax-printing, giving the fabrication process the advantages that lead to high-speed, simplicity, versatility, adaptability and low-cost, is

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best suited for fabricating large numbers (>100) of μPADs in a single batch. In addition, wax is inexpensive, can be obtained anywhere in the world, and is environmentally friendly. However, the traditional wax-printing is accomplished with a wax-printer (Carrilho et al., 2009b; Lu et al., 2009), which is uncommon for developing countries and remote regions. Screen-printing is a printing technique that uses a woven mesh to support an ink-blocking stencil. The attached stencil forms open areas of mesh that transfer ink or other printable materials which can be pressed through the mesh as a sharp-edged image onto a substrate. Screen is made of a piece of porous, finely woven fabric called mesh stretched over a frame of aluminium or wood, and it is low-cost and widely available worldwide. Therefore, in this work, wax-screen-printing was demonstrated by using solid wax as squeegee to force or pump wax past through the pores of the woven mesh to print paper.

Recently, many reported analytical methods have been established on μPADs, such as colorimetric (Martinez et al., 2007; Bruzewicz et al., 2008; Abe et al., 2008; Cheng et al., 2010; Klasner et al., 2010; Li et al., 2010) and electrochemical methods (Nie et al., 2010a; Apilux et al., 2010; Carvalhal et al., 2010; Nie et al., 2010b), electrochemiluminescence (Delaney et al., 2011), absorbance and fluorescence methods (Carrilho et al., 2009a). Besides these works, a sensitive, simple and low-cost microfluidic paper-based chemiluminescence (CL) device has been reported in our previous paper (Yu et al., 2011), in which enzyme biosensors on μPAD were assembled by cutting methods and used for simultaneous detection of glucose and uric acid. However, it is still a critical demand on novel μPADs strategy for accurate quantitation of trace amount of analyte in real complex biological samples. ELISA as an analytical technique is widely applied in the fields of clinical diagnosis (Wild, 2005), drug discovery (Huels et al., 2002), food quality testing (Morris and Clifford, 1985) and environmental safety monitoring (Knopp, 2006). The paper-based ELISA (Cheng et al., 2010), based on the colorimetric assays on paper microzone plate, was firstly proposed by Whitesides and has provided a novel opportunity and protocol for performing highly selective analytical methods on μPADs for real complex biological samples. CL, representing a versatile, ultrasensitive tool with a wide range of applications in biotechnology (Zhang et al., 1999), gives a simple, sensitive and rapid alternative to other methods as a detection principle in ELISA for the POCT of molecules (e.g., proteins, hormones, drugs, nucleic acids and environmental pollutants) (Zhao et al., 2009). To the best of our knowledge, no report about establishing chemiluminescence ELISA (CL-ELISA) on μPADs has been published. In addition, the highly sensitive and selective sandwich method was introduced as a model into this paper-based CL-ELISA in this work.

To perform sandwich CL-ELISA on μPADs, the wet-strength of paper and the stability of the immobilized antibodies on paper are very important. However, an untreated pure cellulose paper will typically lose its strength when saturated in water and offers few functional groups for directly covalent bioconjugation. Chitosan is a linear aminopolysaccharide derived from chitin, one of the most abundant natural polymers and the main component of crustacean shells and insects. Chitosan has been extensively investigated because of its unique properties and applications, such as film-forming ability, biodegradability, antibacterial activity and biocompatibility (Peniche et al., 2008). Therefore, using chitosan, we introduced a novel paper modification strategy for μPADs to simultaneously enhance the wet-strength of μPADs and the stability of immobilized antibodies, because chitosan is readily compatible with paper (due to its structural similarity with cellulose) and imparts the paper products improved mechanical strength (Andersson, 2008; Ashori and Raverty, 2007; Bordenave et al., 2007). And we find that the covalent coupling of antibodies on chitosan modified μPADs possesses high binding-stability and wet-strength for sandwich CL-ELISA on μPADs.

In this paper, using α-fetoprotein (AFP), cancer antigen 125 (CA125) and carcinoembryonic antigen (CEA) and paper microzone plate as model, the simultaneous determination of multiple tumor markers by paper-based CL-ELISA was demonstrated with luminol-*p*-iodophenol-H_2O_2-HRP CL system and showed good analytical performance. In addition, the CL from neighboring zones may interfere with each other. Thus, a spaced-detection strategy was adopted to avoid the CL interference between the neighboring zones. Finally, the concentrations of AFP, CA125 and CEA in real human serum samples were detected under the optimum conditions. And the reusability, reproducibility and stability were investigated. This paper-based CL-ELISA will not only be very useful when the level of an analyte in real biological sample is important for POCT, public health and environmental monitoring in remote regions, developing or developed countries, but also make contribution to further expand functional, analytical and fabricating protocol on μPADs.

2. Material and methods

2.1. Reagents and materials

All mouse monoclonal AFP, CA125 and CEA capture antibodies, HRP-labeled AFP, CA125 and CEA signal antibodies, fluorescein isothiocyanate (FITC)-labeled AFP, CA125 and CEA antibodies and standard AFP, CA125, CEA solutions were obtained from Linc-Bio Science Co. Ltd. (Shanghai, China). Chitosan (85% deacetylation), glutaraldehyde and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Luminol-*p*-iodophenol-H_2O_2 solution used as HRP CL substrate was supplied by Autobio Diagnostics Co. Ltd. Whatman chromatography paper #1 (WCP#1) (20 cm × 20 cm) (pure cellulose paper) was obtained from GE Healthcare Worldwide (Pudong Shanghai, China) and used with further adjustment of size. Ultrapure water obtained from a Millipore water purification system (g18 MΩ, Milli-Q, Millipore) was used in all assays and solutions. Coupling buffer for antibody immobilization was 0.01 mol L^{-1} pH 7.4 phosphate buffer solution (PBS). Blocking buffer for the residual reactive sites on the antibody immobilized paper was PBS containing 0.5% bovine serum albumin and 0.5% casein. 0.05% Tween-20 was spiked into 0.01 mol L^{-1} pH 7.4 PBS as wash buffer (PBST) to minimize unspecific adsorption. The clinical serum samples were from Shandong Tumor Hospital. All other reagents were of analytical grade and used as received.

2.2. Wax-screen-printing of the paper microzone plate

The schematic representation of the paper microzone plate fabricated by wax-screen-printing was shown in Scheme 1. The model paper microzone plate in this work, arranged in an array of 6 columns by 3 rows, has a diameter of 6 mm with a center-to-center distance of 8 mm. Hydrophobic barriers as black zones on a white background were designed using Adobe Illustrator software (Adobe Systems, Inc.). The designed microzone plate pattern was used to produce the screen at a local printing shop. As shown in Scheme 1, firstly, the screen (200 mesh of nylon on an aluminium frame) is placed on paper surface closely, and a solid wax, used as squeegee, is rubbed through the screen stencil, forcing wax past the pores of the woven mesh to form wax patterns on paper surface. Afterwards, the wax-screen-printed paper was placed in an oven set at 130 °C for 150 s, and then the wax melted and penetrated the thickness of the paper (Scheme 1) to create complete hydrophobic barriers in paper that formed the microzones. The wax-penetrated paper was ready for use after removing the paper from the hot plate and allowing it to cool to room temperature (<10 s). The screen