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An integrated electrochemical device based on immunochromatographic test strip and enzyme labels for sensitive detection of disease-related biomarkers

Zhe-Xiang Zou[a,b], Jun Wang[b], Hua Wang[b], Yao-Qun Li[a,*], Yuehe Lin[b,**]

[a] Department of Chemistry and Key Laboratory of Analytical Sciences, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, Fujian Province, China
[b] Pacific Northwest National Laboratory, Richland, WA 99352, USA

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ABSTRACT

A novel electrochemical biosensing device that integrates an immunochromatographic test strip and a screen-printed electrode (SPE) connected to a portable electrochemical analyzer was presented for rapid, sensitive, and quantitative detection of disease-related biomarker in human blood samples. The principle of the sensor is based on sandwich immunoreactions between a biomarker and a pair of its antibodies on the test strip, followed by highly sensitive square-wave voltammetry (SWV) detection. Horseradish peroxidase (HRP) was used as a signal reporter for electrochemical readout. Hepatitis B surface antigen (HBsAg) was employed as a model protein biomarker to demonstrate the analytical performance of the sensor in this study. Some critical parameters governing the performance of the sensor were investigated in detail. Under optimal conditions, this sensor was capable of detecting a minimum of 0.3 ng mL^{-1} (S/N = 3) HBsAg with a wide linear concentration range from 1 to 500 ng mL^{-1}. The sensor was further utilized to detect HBsAg spiked in human plasma with an average recovery of 91.3%. In comparison, a colorimetric immunochromatographic test strip assay (ITSA) was also conducted. The result shows that the SWV detection in the electrochemical sensor is much more sensitive for the quantitative determination of HBsAg than the colorimetric detection, indicating that such a sensor is a promising platform for rapid and sensitive point-of-care testing/screening of disease-related biomarkers in a large population.

1. Introduction

It is of great importance to rapidly and sensitively detect disease-related biomarkers that serve as indicators of biological and pathological processes, or physiological and pharmacological responses to a drug treatment. Since 1959 when Yalow and Berson [1] first introduced radioisotope-labeled immunoassays, immunoassays have acted as one of the most popular approaches for detection of biomarkers due to its high specificity, simplicity, and versatility [2–5]. Besides, during the past years, other approaches such as microsphere-based arrays [6], proteome chips [7], surface plasma resonance [8], microfluidic systems [9], surface-enhanced Raman spectroscopy [10,11] have also been developed for biomarker detection. Despite their success on providing precise results and reliable predictions, these approaches still suffer from long analysis time and complexity due to multiple-step processes and the requirements of laboratory-oriented instruments, which limits their further applications in in-field or point-of-care (POC) diagnosis. As a result, it is essential to develop a rapid, simple, and cost-effective approach as a tool complementary to those traditional methods to meet the increasing requirement of screening disease-related biomarkers in large group of people.

Immunochromatographic test strip assays (ITSAs), in combination of the excellent separation ability of chromatography with the high specificity and sensitivity of conventional immunoassay, opens up a new avenue for protein analysis and clinical diagnosis [12–15]. The advantages of ITSAs include easy operation, short analysis time, less interference and relatively low cost. Moreover, it is one-step analysis and portable. Thus, ITSAs can serve as an ideal tool for detection of various biomarkers of diseases in human bodies. The early generations of ITSAs were usually based on eye-detection (colorimetric), wherein gold nanoparticles as well as some organic dyes were used for signal readout [16–21]. Although these ITSAs are still very popular nowadays due to their simplicity, they can only provide qualitative or semi-quantitative results, like yes/no answers, and the concentrations of the analytes in the sample should be high enough. This is not adequate for ultrasensitive

* Corresponding author. Tel.: +86 592 2185875; fax: +86 592 2185875.
** Corresponding author.
E-mail addresses: yqlig@xmu.edu.cn (Y.-Q. Li), yuehe.lin@pnl.gov (Y. Lin).

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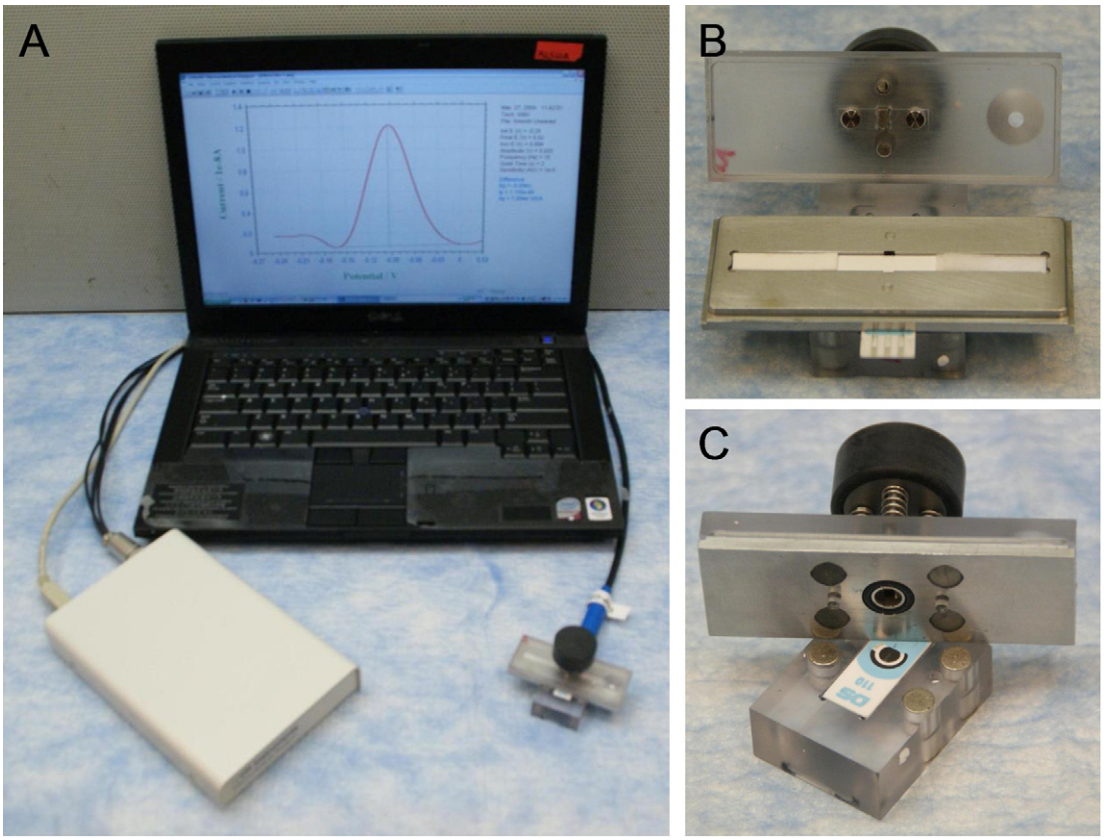

Fig. 1. Photograph of (A) the entire sensor system including a portable IEB device with the test strip and the SPE, a portable electrochemical analyzer connected to a laptop, (B) the IEB device containing a chamber where a test strip is housed and (C) the IEB containing an electrochemical cell where a SPE is placed.

screening of disease-related biomarkers in early stage because the concentrations of the biomarkers are often very low at this time and the information of the biomarkers is very important for disease diagnosis. Therefore, more quantitative ITSA should be developed to provide the accurate level of the biomarkers in biological fluids.

Currently, optical [22–24], electrochemical [25,26], and magnetic [27] readout systems have been employed as signal transducers to develop such quantitative ITSAs. Electrochemical immunoassays and electrochemical immunosensors have gained considerable attentions and evolved dramatically over the past two decades. For example, electroactive-species loaded liposome has been used to detect theophylline [25] and metal ion chelates have been used to detect human chorionic gonadotropin (HCG) [26]. Recently, inorganic nanoparticles such as gold nanoparticle [28,29] and quantum dots [14] have also been introduced into electrochemically quantitative ITSAs to amplify the signal. However, these methods require the dissolutions of inorganic nanoparticles, involving in unfavorable harsh acid which is harmful to the electrode. Another disadvantage is that the mobility of the inorganic nanoparticles is usually very poor on the test strips, requiring tedious treatments to the membrane to avoid non-specific adsorptions.

Horseradish peroxidase (HRP) is one of the most useful and important peroxidase enzymes used in a wide variety of bioassays as a label. It can produce colored, fluorimetric, luminescent or electroactive enzymatic products by catalytic oxidation of a substrate, therefore, allowing the analytes to be detected and quantified using optical or electrochemical techniques [30]. HRP is often used in conjugates to determine the presence of a molecular target. For example, an antibody conjugated to HRP may be used to detect a small amount of a specific protein in a western blot [31]. Other applications of HRP include enzyme-linked immunosorbent assay (ELISA) [3], immunohistochemistry [32], in situ hybridization [33], immunosensors [34] and DNA biosensors [35]. Common HRP substrates developed for HRP-labeled electrochemical bioassays include 3,3′5,5′-tetramethylbenzidine (TMB) [36], o-phenyldiamine (OPD) [37], hydroquinone [38], hydroxymethyl ferrocene [39], osmium complex [40]. The use of HRP in electrochemical immunoassay allows excellent solubility in the solution and good mobility on the test strip, as well as avoiding unfavorable harsh acid. Moreover, the signal can be amplified by enzymatic reactions, thus greatly improving the sensitivity of the ITSA. Recently, Lin and co-workers [41] have demonstrated a novel portable enzyme-linked integrated electrochemical biosensor (IEB) for simple, rapid, and sensitive biomonitoring of trichloropyridinol, a metabolite biomarker of exposure to organophosphorus insecticides. However, such enzyme-linked IEB has not yet been employed for quantitative detection of disease-related biomarkers.

In this article, we present an IEB device based on immunochromatographic test strip and enzyme labels for simple, sensitive, and quantitative detection of disease-related biomarkers in human plasma. We used hepatitis B surface antigen (HBsAg) as a model protein biomarker because it is the earliest indicator of acute hepatitis B and frequently used for identifying infected people before symptoms appear [42–46]. The early diagnosis of HBsAg combined with the effective treatment offers the best chance of recovery. Some commercial ITSAs have been reported for detecting HBsAg [47,48]. However, most of ITSAs for HBsAg detection were still limited in qualitative analysis based on eye-detection (colorimetry), which can only provide positive or negative results. This is not adequate when the level of HBsAg is required. The approach developed in this work combines the advantage of the ITSA and the high sensitivity and the simplicity of enzyme-catalyzed electrochemical

detection, resulting in a novel, portable, and rapid sandwich immunoassay tool for sensitive and selective detection of HBsAg. Therefore, this sensor platform may open up a new avenue for rapid POC screening of disease-related biomarkers and clinical diagnosis.

2. Experimental

2.1. Chemicals and materials

Phosphate buffer saline (PBS, 0.01 M, pH 7.4), bovine serum albumin (BSA), Tween-20, OPD, hydrogen peroxide (30%, H_2O_2), 3,3-diaminobenzidine (DAB, with diluent) and human plasma (sodium citrate treated) were purchased from Sigma–Aldrich. HBsAg (2.5 mg mL^{-1}), mouse monoclonal HBsAg antibody (1 mg mL^{-1}), HRP labeled rabbit polyclonal HBsAg antibody (1 mg mL^{-1}) were purchased from Medistar Biotech Inc., Canada. Nitrocellulose membrane, absorbent pad, sample pad, and conjugation pad as well as backing cards were purchased from Millipore (Temecula, CA). All chemicals used in this study were of analytical grade. All stock solutions were prepared using deionized water purified with the Nanopure System (Barnstead, Kirkland, WA). Sample dilution buffer as well as washing buffer was prepared by dissolving appropriate amount of BSA and Tween-20 in PBS to form a final buffer containing 3% BSA and 0.02% Tween-20 in 0.01 M PBS (PBSBT). Electrochemical substrate for square-wave voltammetry (SWV) detection consists of 0.2 mM OPD and 0.4 mM H_2O_2. DAB was 10 times diluted from the original stock solution by its diluent as color solution. The original HBsAg stock solution was diluted to serial concentrations by PBSBT buffer for further experiments.

2.2. Design and fabrication of an integrated electrochemical biosensor (IEB)

The IEB device used in this study is shown in Fig. 1. This device consists of a chamber in which a test strip is housed and an electrochemical cell where a screen-printed electrode (SPE) is placed. The chamber is on the top of the electrochemical cell and there is a channel connecting the chamber to the electrochemical cell. The electrochemical cell is formed by a plastic O-ring between the chamber and the bottom base. The O-ring is used to seal the electrolyte solution by built-in magnet in the chamber and the bottom base. The test zone of the strip is located in the channel. On the top of cover is placed a cutter. Once the completion of the immunoreactions on the test strip, the cutter will be pressed down and punch the test strip membrane, resulting in the test zone membrane falling down to the electrochemical cell. Following that, electrochemical measurements will be carried out for quantification of protein biomarkers in the samples.

2.3. Instruments

The test strip fabrication system consists of a XYZ-3050 dispenser, LM5000 laminator and the Guillotine cutting system CM 4000 which were purchased from BioDot LTD (Irvine, CA). The XYZ-3050 dispenser includes AirJet Quanti 3000 dispenser and BioJet Quanti 3000 dispenser. All SWV measurements were carried out with a portable electrochemical analyzer CHI1324 (CH Instruments, Inc., Austin, TX) connected to a laptop computer (Fig. 1A). SWV scanning was performed from −0.25 to 0.05 V with a step potential of 4 mV, an amplitude of 25 mV and a frequency of 15 Hz. Baseline corrections were carried out using CHI software. The peak value of the current near the potential of −0.02 V was used as the response of the IEB. Disposable SPE consisting of a carbon working electrode, a carbon counter electrode, and an Ag/AgCl reference electrode (Dropsens, Inc., Spain) were used for electrochemical measurements. A sensor connector (Dropsens Inc., Spain) was used to connect SPE to the CHI electrochemical analyzer. A novel and smart device consisting of a chamber and an electrochemical cell was fabricated for housing the test strip (Fig. 1B) and the SPE (Fig. 1C), respectively.

2.4. Test strip preparation

The test strip consists of five components: sample application pad, conjugate pad, nitrocellulose membrane, absorbent pad and backing card. Preparation of HBsAg test strip was described as follows. The sample application pad (20 mm × 30 cm) and the conjugation pad (8 mm × 30 cm) were both made of glass fiber. A desired volume of 1 μg mL^{-1} HRP-antibody conjugate solution was dispensed on the conjugate pad with the dispenser XYZ-3050 BioJet Quanti 3000 and was dried at 4 °C for 1 h, and was stored in the same condition. The test zone of the strip was prepared by dispensing a desired volume of 1 mg mL^{-1} mouse monoclonal HBsAg antibody solution with the dispenser XYZ-3050 BioJet Quanti 3000 onto nitrocellulose membrane (40 mm × 30 cm), and was dried for 1 h at 4 °C. Both the sample pad and the absorbent pad (20 mm × 30 cm) were stored in room temperature without any treatments. All of the above four parts were assembled on a plastic adhesive backing card (60 mm × 30 cm) using the batch laminating system LM5000. Each part overlapped 2 mm to ensure the solution migrating through the strip during the assay. Finally, the HBsAg test strips with a 4 mm width were cut by using the Guillotine cutting system CM 4000 and assembled in a compact device (Fig. 1B) for the testing.

We have investigated the biological activity of antibodies on test strips by periodical testing of ITSAs using HBsAg samples and found that the activity of antibodies in test zone can maintain up to 6 months if stored properly (4 °C, sealed).

2.5. Electrochemical immunochromatographic assay of HBsAg

The sandwich immunoassay on the test strip was performed as follows: 60 μL of sample solution in PBSBT buffer containing desired concentrations of HBsAg were added to the sample application pad. PBSBT buffer or plasma without HBsAg was used as control. After an adequate incubation (e.g. 15 min), the immunoreactions completed and the sandwich complex (HRP-antibody)–HBsAg-(capturing antibody), formed on the test zone. Subsequently, in order to reduce the non-specific adsorption on the membrane, 60 μL of PBSBT buffer were used to wash the test strip in the similar way as the above operation. Then, the test zone was cut down and fell onto the electrochemical cell where the SPE is integrated, followed by adding 50 μL of electrochemical substrate. After certain time (e.g. 10 min) of enzymatic reaction, SWV measurement was carried out on electrochemical analyzer.

3. Results and discussion

3.1. Principle of immunoreactions on the test strip

Fig. 2 illustrates the principle of the ITSA, which is based on sandwich immunoreactions on the test strip between HRP-antibody, analyte and capturing antibody. Certain amount of analyte solution is first applied to the sample application pad (Fig. 2A). Then the capillary force causes the liquid sample to migrate towards the other end of the strip. As the liquid sample migrates into the conjugate pad, the immunoreactions between the analytes and the HRP-antibody conjugates occur and the (HRP-antibody)–analyte complexes form. The mixtures of the complexes and the excess HRP-antibodies continue to migrate along the strip by capillary force (Fig. 2B). When these mixtures reach the test zone, the complexes of (HRP-antibody)–analyte bind to capturing antibodies

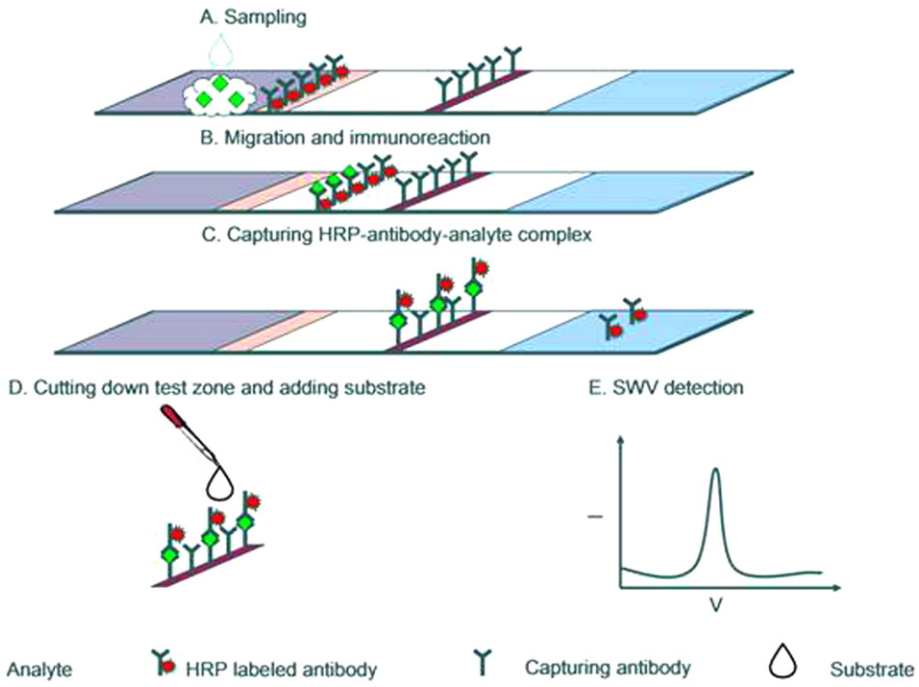

Fig. 2. Schematic illustration of principle of electrochemical ITSA at the IEB. (A) Aqueous sample containing analytes was applied to sample zone. (B) Analytes flow through the conjugation pad and the (HRP-antibody)–analyte complex forms. (C) The (HRP-antibody)–analyte complex is captured by the capturing antibody and the final complex, the (HRP-antibody)–analyte-(capturing antibody), forms. Excess HRP-antibodies continue to migrate towards the absorption pad. (D) The test zone is cut down and the electrochemical substrate is added. (E) SWV detection is carried out at IEB with a portable electrochemical analyzer.

which are immobilized on the test zone through immunoreactions between the analytes and the capturing antibodies to form the final sandwich complex, (HRP-antibody)–analyte-(capturing antibody). The fluid fraction containing excess HRP-antibodies continues to flow into the absorbent medium at the end of the strip (Fig. 2C). In a control assay (no analyte), almost no HRP-antibodies bind to the capturing antibodies in the test zone. After a complete immunoassay, the test zone is cut down by pressing down the cutter on the top of the device. The test zone strip will fall down into the electrochemical cell and to which certain amount of electrochemical substrate is added (Fig. 2D). Subsequently, the resulting solution is subjected to SWV detection with an electrochemical analyzer (Fig. 2E). The more analytes in the sample, the more HRP-antibodies would bind to the capturing antibodies in test zone through the sandwich immunoreactions, which leads to higher electrochemical signals. Therefore, the electrochemical signal is proportional to the analyte concentrations in sample, which can be used for quantification of HBsAg in samples.

3.2. Optimization of ITSAs parameters

To achieve the optimal performance of the sensor, three critical parameters including the buffer washing step, the immunoreaction time and the amount of the sample applied to the test strip were investigated. These parameters can effectively affect the response of the sensor and background signals.

The non-specific adsorption is a common problem in most immunoassays, as well as in the test trip assays. Thus, the effect of buffer washing after immunoreactions was firstly investigated. Two parallel series of test strip assays were designed, wherein one was applied only to HBsAg sample solutions without buffer washing step while the other introduced an additional buffer washing step after applied with the same sample solutions. As can be seen from Fig. 3, in the series without washing step, there was no obvious linear relationship between the signals and HBsAg concentrations since the signals fluctuated seriously. This was caused by non-specific adsorption of abundant free HRP-antibody conjugate on the membrane. In contrast, the situation for the series with an additional washing step is completely different. It is obvious for this series that electrochemical signals increased with the increase of HBsAg concentrations, suggesting that such buffer washing step can effectively reduce non-specific adsorption of HRP-antibody conjugates on the test membrane. Therefore, the washing step is necessary for minimizing the non-specific adsorption on the test strips.

The amount of a sample applied to the test strip was also optimized. A series of 30, 40, 50, 60 and 80 μL of the solutions containing 250 ng mL^{-1} HBsAg were applied to the test strips, respectively. The SWV signals were plotted versus the amount of sample. As shown

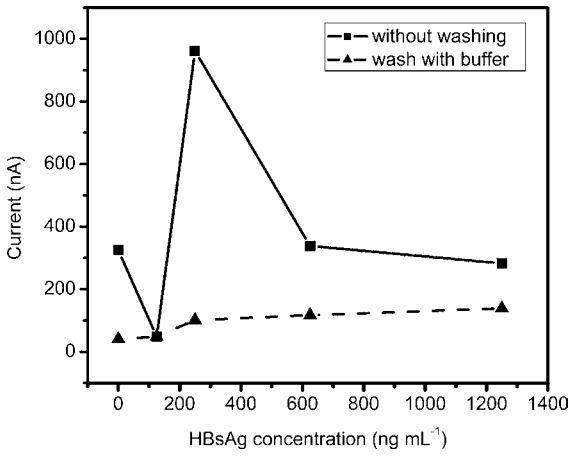

Fig. 3. Effect of buffer washing on electrochemical response. The concentrations of HBsAg were 156, 250, 625 and 1250 ng mL^{-1} in PBSBT. The amount of sample applied was 60 μL and the immunoreaction time was set to 15 min.

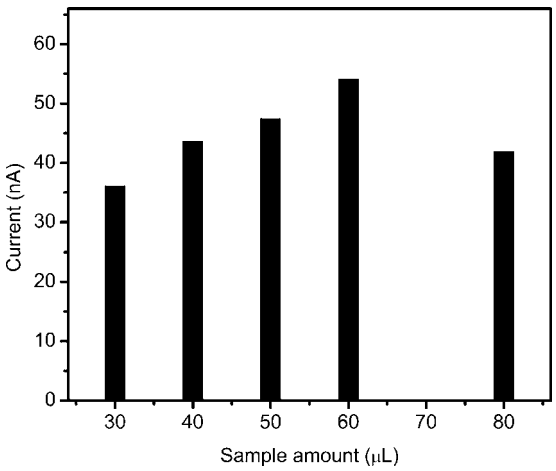

Fig. 4. Effect of the amount of sample applied to the test strip on electrochemical response. The concentration of HBsAg was 250 ng mL^{-1}. PBSBT buffer was used as washing buffer. The immunoreaction time was 15 min.

in Fig. 4, the signal gradually increased from sample amount of 30 to 60 μL, then decreased at 80 μL. Normally, as the sample amount increases, the amount of the complex of (HRP-antibody)–analyte-(capturing antibody) will increase accordingly, resulting in signal increases. However, too large amount of the sample, such as 80 μL, will lead to signal decrease. This may be due to the fact that the free HBsAg will bind to capturing antibodies in the test zone in the case of large sample amount, which may limit the binding between the complex of (HRP-antibody) and the capturing antibodies, thus cause signal decrease. According to the above result, 60 μL of sample amount was applied for further experiments.

Another parameter affecting the electrochemical response of the sensor is the immunoreaction time. We studied the effect of three different immunoreaction times on the electrochemical signal. With the immunoreaction time of 10 min, the signal deviations are very high and the signal ratio of sample to control is low. This suggests that the immunoreaction has not completed and there should be non-specific adsorption. When the immunoreaction time increases above 15 min, the signal deviation becomes very low, indicating that the immunoreaction has completed. The signal ratio of sample to control showed the highest peak at 15 min. Considering the time consumption and signal intensity, 15 min was used for the following experiments.

3.3. Analytical performance of the sensor

The electrochemical ITSA were performed at the IEB under the optimal conditions to evaluate the feasibility of the sensor for rapid and sensitive detection of HBsAg. A series of HBsAg solutions with concentrations of 1, 5, 10, 50, 100, 500 and 1000 ng mL^{-1} were prepared and were applied to the test strips, respectively. It was found that the electrochemical response constantly increased with the increase of the concentration of HBsAg. A calibration curve was obtained and shown in Fig. 5A by plotting the peak value of the current versus the concentrations of HBsAg. It was found that the sensor had a wide linear range from 1 to 500 ng mL^{-1} ($y = 0.12x + 37.13$, $R = 0.9982$). A detection limit was estimated about 0.3 ng mL^{-1} based on S/N = 3. Such results demonstrate that the sensor can be used to sensitively detect HBsAg because HBsAg generally circulates at concentrations of 50–300 μg mL^{-1} in chronic carriers and that values below 20 ng mL^{-1} are rarely observed [49] though the lowest reported HBsAg dose in an asymptomatic blood donor is 0.2 ng mL^{-1} [50].

3.4. Evaluation of the sensor with in vitro human plasma samples

To validate the sensor for its clinic application, this electrochemical sensor was further evaluated with a human plasma sample spiked with standard HBsAg. The plasma samples were spiked with 10, 50, 100 and 250 ng mL^{-1} HBsAg and were directly tested at the IEB without any further treatments. The plasma sample without spiking HBsAg serves as a control. As shown in Fig. 5B, a similar linear relationship between the electrochemical response and the concentrations of HBsAg spiked was observed. The concentrations of HBsAg spiked in plasma were calculated based on the electrochemical response and the calibration curve obtained in the matrix of healthy human plasma (containing no detectable HBsAg). The

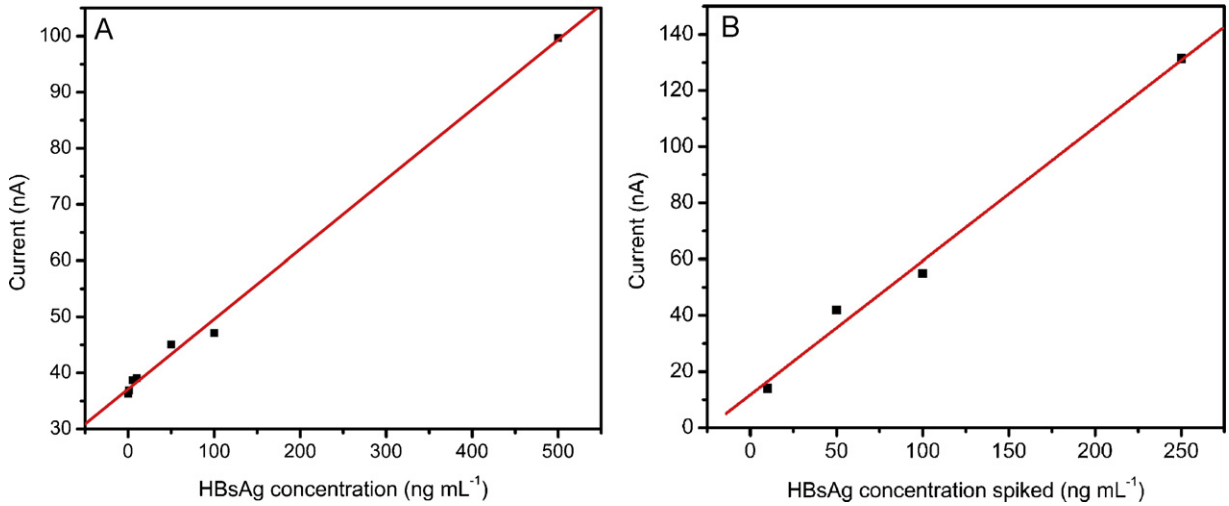

Fig. 5. (A) Calibration curve obtained at the IEB device with standard solutions of HBsAg: 1, 5, 10, 50, 100 and 500 ng mL^{-1}, respectively. PBSBT buffer was used as a control, as well as washing buffer. The amount of sample applied to the test strip was 60 μL and the immunoreaction time was set to 15 min. (B) Electrochemical responses obtained at the IEB device with increasing the concentration of HBsAg spiked in plasma. The spiked plasma was directly applied to the device for analysis without any further treatments such as dilution. The plasma without spiking HBsAg served as a control. The HBsAg standard was spiked into the plasma to obtain final concentrations of 12.5, 50, 100 and 250 ng mL^{-1}. The immunoassay conditions were the same as in (A).

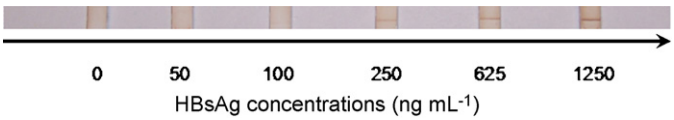

Fig. 6. Typical photographs of ITSAs with colorimetric detection. 20 μL 10-folded diluted DBA color solution was applied to the test zone on the membrane after the immunoreaction had completed and the photo was taken after 5 min of color generation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

average recovery of HBsAg spiked in plasma was 91.3%, indicating that the electrochemical sensor is capable for rapid, accurate, and sensitive determination of HBsAg in human plasma samples, thanks to the excellent separation ability of immunochromatographic test strip.

3.5. Colorimetric detection of ITSA

To date, colorimetric detection is still the most popular signal readout system for ITSAs since its simplicity and convenience. We performed colorimetric detection by adding 20 μL diluted DAB solution to the test zone on the membrane after the immunoreactions were completed. The results were shown in Fig. 6. It can be seen from this figure that brown test lines appeared on the strips when HBsAg concentration is higher than (including) 100 ng mL^{-1}, while no test lines appeared on those strips when HBsAg concentration is lower than 100 ng mL^{-1}. Moreover, the color of test lines displayed clear gradient according to HBsAg concentrations, which indicates that this colorimetric detection is hopeful to be a semi-quantitative determination method when HBsAg concentration is above 100 ng mL^{-1}. The color of the test line on the test strip with 100 ng mL^{-1} was very weak, indicating that the detection limit of this colorimetric method is approximately 100 ng mL^{-1} which is extremely high compared with electrochemical detection.

4. Conclusion

We have successfully developed a novel integrated electrochemical device based on a lateral flow test strip and an enzyme label for simple, rapid and quantitative detection of HBsAg in human blood samples. The electrochemical sensor takes the advantages of the speed and low cost of conventional immunochromatographic strip test and high sensitivity of the enzyme-based electrochemical immunoassay. Under optimal conditions, this sensor is capable of detecting a minimum of 0.3 ng mL^{-1} HBsAg and detecting HBsAg spiked in human plasma with an average recovery of 91.3%. Due to its excellent separation ability, HBsAg in plasma could be directly determined by this sensor. The compact electrochemical device coupled with a portable electrochemical analyzer would be a promising tool for rapid and sensitive POC testing and screening of disease-related biomarkers in large group of people.

Acknowledgments

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