Moving Enzyme-Linked ImmunoSorbent Assay to the Point-of-Care Dry-Reagent Strip Biosensors

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Abstract

In this work, we described a point-of-care (POC) dry-reagent strip biosensor (DRSB) based on enzyme tracers and portable strip reader for simple, low-cost and sensitive assay of protein detection in minutes. Horseradish Peroxidase (HRP) and Rabbit IgG (R-IgG) were used as a model system for the demonstration of the proof-of-concept. The sandwich-type immunoreactions were performed on the DRSB and the HRP tracers were captured on the test zone of the biosensor. The excess of HRP tracers were captured on the control zone of the biosensor through the immobilized secondary antibody. Subsequent enzymatic reaction in the presence of the substrate produced insoluble enzymatic products, which deposited on both test and control zones of the DRSB and formed two characteristics blue bands. While qualitative tests are realized by observing the color change of the test zone, quantitative data are obtained by recording the intensities of the test zone with a portable "strip reader". The quantitative response of the optimized DRSB over the range of 0.1-50 ng mL^{-1} IgG in association with a 10-min assay time is obtained, and the limit of detection is estimated to be 0.05 ng/mL, which is ten times lower than that of the gold nanoparticle (GNP)-based DRSB. The enzyme-based DRSB was used to detect Carcinoembryonic Antigen (CEA) biomarker in human plasma successfully. Such enzyme-based DRSB offers a simple and fast tool for point-of-care protein assay and a potential substituent for the traditional Enzyme-linked Immunosorbent Assay (ELISA).

Keywords: ELISA; Biosensor; Enzyme; Point-of-care; Dry-reagent strip.

1.0 Introduction

Immunoassays, as promising approaches for selective and sensitive analyses, have recently gained increasing attention in different fields including food safety, environmental attention and clinical diagnosis. In clinical diagnosis, the quantification of extremely low concentration of proteins play pivotal roles on both diagnosis and treatment of diseases, the detection of infectious agents, and on warning against bio-warfare agents [1, 2]. One of the most widely used methods for the detection of proteins is Enzyme-Linked Immunoabsorbent Assay (ELISA) [3], which is performed on the 96 multi-well plates. Following a sandwich-type or competitive immunoreactions in the micro-wells, the captured enzyme traces are quantified by the measurement of the absorbance of enzymatic products in the presence of specific substrates. Because of its high sensitivity and multiplex capabilities, ELISA has become a household name for medical laboratories, manufactures of *in vitro* diagnostic products, regulatory bodies, and external quality assessment and proficiency-testing organizations [3]. However, tedious assay procedures, extended time and requirement of highly qualified personnel limit its applications in point-of-care (POC) and in-field detection [4]. Although automated ELISA instruments have been introduced to overcome the disadvantages of the traditional ELISA, yet the cost still is a big concern.

Immunochromatographic strip tests (IST) combine chromatography technology with conventional immunoassay to offer a simple, low-cost and fast tool for protein analysis and clinical diagnosis [5-8]. Compared with ELISA, such IST has several advantages: user-friendly format, very short assay time (generally several minutes), less interference due to chromatographic separation, long-term stability over a wide range of climates, a relatively low cost, and no requirements for skilled technicians. This ideal technique is suitable for on-site testing by people who are untrained analysts. The IST was utilized initially for POC testing of symptoms such as pregnancy and ovulation and, more recently, for the diagnosis of various diseases and environmental monitoring [9-11]. The major limitations of such colloidal gold based IST are the relatively low-sensitivity and just qualititative or semi-quantitative concentration information of the target protein could be obtained [12]. Combining the high sensitivity of ELISA and the unique above mentioned advantages of the IST, the enzyme based immunochromagographic strip test (EIST) has shown the promising to detect proteins at very low concentration levels. The EIST was pioneered by different groups [13-17]. Early EIST was based on the measurement of the height of the formed color bands of the enzymatic product on the strip [13, 14]. The performance of the EIST was improved by designing cross-flow plastic chips and analysis of the intensities of the formed bands [16, 17]. However the quantification of the analytes was usually performed on either the array scanner or complex and expensive instrumentations, which limits its in-field and POC applications. Recently, we reported quantitative dry-reagent strip biosensors (DRSB) for the detection of protein and nucleic acids based on a portable strip reader and gold nanoparticle probes [18, 19]. The portable strip reader is a sensitive image analyzer to read color intensity of bands on the membrane. It is a powerful tool for POC and in-field detection in connection to an ultralight lab-top computer. Its software could search lines in fixed reaction area automatically and figure out parameters such as peak height, line distance, integral area etc.

In this work, we report on an enzyme-based DRSB in connection with the portable strip reader for POC and in-field detection of very low concentration of protein. We intended to move the traditional ELISA to the portable DRSB. The proof-of–concept was demonstrated firstly by using an IgG model system. Comparing with the GNP-based DRSB, the sensitivity of enzyme-based DRSB increased 10 times and it was capable of detecting 0.05 ng mL^{-1}IgG in 10 min. The feasibility of such enzyme-based DRSB was evaluated by detecting Carcinoembryonic Antigen (CEA) in human plasma. The promising properties of the biosensor are reported in the following sections.

2.0 Materials and methods

2.1. Apparatus

Airjet AJQ 3000 dispenser, Biojet BJQ 3000 dispenser, Clamshell Laminator and the Guillotine cutting module CM 4000 were from Biodot LTD (Irvine, CA). Centrifuge 5415D was supplied from Eppendorf, Hamburg, Germany. Portable strip reader DT1030 was purchased from Shanghai Goldbio Tech. Co., LTD (Shanghai, China).

2.2. Reagents

Polyester backing materials, Glass fibers (GFCP000800), cellulose fiber sample pads (CFSP001700), laminated cards (HF000MC100) and nitrocellulose membranes (HFB24004) were provided from Millipore (Bedford, MA). Horseradish Peroxidase (HRP) conjugated Polyclonal ImmunoPure® Goat Anti-Rabbit IgG (H+L), ImmunoPure® Polyclonal Goate anti-rabbit IgG, rabbit IgG (R IgG) and human IgM were purchased from Thermo Scientific. HRP conjugated monoclonal antibody against human Carcinoembryonic Antigen (CEA), polyclonal antibody to human CEA and human CEA were purchased from Fitzgerald Industries International (Concord MA). Phosphate buffer saline (PBS; 0.01 M), $HAuCl_4$, sodium citrate, phenoxyacetic acid, bovin serum albumin (BSA), hydrogen peroxide, 4-chloro-1-naphthol and Tween-20 were purchased from Sigma-Aldrich. All chemicals used in this study were analytical reagent grade. All stock solutions were prepared using deionized water purified with the Nanopure System (Barnstead, Kirkland, WA). All buffer solutions were prepared using ultrapure (>18 MΩ cm) water from a Millipore Milli-Q water purification system (Billerica, MA).

2.3. Fabrication of Enzyme-based Dry-Reagent Strip Biosensor (DRSB)

The structure of DRSB is similar with that of traditional lateral flow test strip and consisted of the following components: sample application pad, conjugate pad, nitrocellulose membrane and absorption pad (see the scheme in **Figure S1**). The sample application pad (17 mm × 30 cm) was made from cellulose fiber and was soaked with a buffer (pH 8.0) containing 0.25% Triton X-100,

0.05 M Tris-HCl and 0.15mM NaCl. Then it was dried and stored in desiccators at room temperature. The conjugate pad was prepared by two times dispensing of a desired volume of diluted HRP conjugated Polyclonal ImmunoPure® Goat Anti-Rabbit IgG solution (for example, 1:10, the diluted conjugate solution was prepared in the buffer containing 20 mM Na_3PO_4, 5% BSA, 0.25% Tween and 10% sucrose) onto the glass fiber pad and then drying it at room temperature. The pad was stored in a desiccator at 4 ℃. A concentration of 1.2 mg/mL polyclonal rabbit anti-IgG and goat anti-rabbit IgG solutions were dispensed on the different location of the nitrocellulose membrane (25 mm × 30 mm) as test zone and control zone by using Biojet BJQ 3000 dispenser. The nitrocellulose membrane was then dried at 37 ℃ for 1 h and stored at 4 ℃ in dry conditions. Finally, all of the parts were assembled on a plastic adhesive backing layer (typically an inert plastic, e.g., polyester) using the Clamshell Laminator (Biodot, CA, USA). Each part overlapped 2 mm to ensure the solution migrating through the biosensor during the assay. The DRSBs with a 4.7 mm width were cut by using the Guillotin cutting module CM 4000. The DRSB was secured in a cassette that is composed of a top cover and an adapted bottom base.

In the case of the preparation of the enzyme-based DRSB for the detection of CEA, the diluted HRP conjugated monoclonal antibody against CEA solution was dispensed on the conjugate pad, and both polyclonal rabbit anti-human CEA and polyclonal goat anti- rabbit IgG were dispensed on the nitrocellulose membrane as test and control zones, respectively. Other procedures are same as the above description.

2.4. Fabrication of GNP-based DRSB

GNPs with average diameter 20 ± 3.5 nm were prepared according to the reported methods [20] with slight modifications. GNP–anti-IgG conjugates were prepared following the reported protocol [21]. The GNP-anti-IgG conjugate solution was dispensed on the glass fiber pad. Other preparations of GNP-based DRSB are following the procedure described above.

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2.5. Sample Assay Procedure

One-hundred and twenty microlitters of sample solution containing a desired concentration of rabbit IgG was applied to the sample application zone. After waiting for a desired time (for example, 5 min), additional 25 µL of running buffer (PBS +2%BSA) was added to the sample pad. Following 3 minutes' waiting, 20 µL of the substrate solution containing 8.4 mM 4-chloro-1-naphthol and 4 mM H_2O_2, which was freshly prepared in phosphate buffer solution (0.01M, pH 7.4) containing 15% methanol, was applied to the sample pad. The enzymatic reaction proceeded for 2 min to deposit insoluble enzymatic product (4-chloro-1-naphthon) on the test zone and control zone of the DRSB, showing two blue bands on the membrane. For quantitative measurements, the DRSB was inserted into the strip reader DT1030, where the optical intensities of both test and control lines could be recorded simultaneously by using the "GoldBio strip reader" software.

The detection of CEA with the enzyme-based DRSB in human plasma is similar with the above procedure with a little modification, in which 25 µL of plasma was applied on the sample pad, and 105 µL running buffer was added to push the sample through the DRSB.

3. 0 Results and Discussion

3.1. Principle of the Enzyme based DRSB for POC Test

The proof-of-concept of the enzyme based DRSB for POC test was demonstrated firstly by using an IgG model system. A pair of antibodies including HRP conjugated polyclonal goat anti-rabbit IgG (HRP-anti-IgG) and polyclonal goat anti-rabbit IgG (anti-IgG) were used to prepare the DRSB. The HRP-anti-IgG conjugate was immobilized on the conjugate pad and anti-IgG was immobilized on the test zone of nitrocellulose membrane as capturing antibody. The secondary antibody against the anti-IgG was immobilized on the control zone of the nitrocellulose membrane. In a typical assay, a sample solution containing a desired concentration of IgG was applied to the sample application pad. The solution migrates along the membrane by capillary force and rehydrates the HRP-anti-IgG in the conjugate pad.
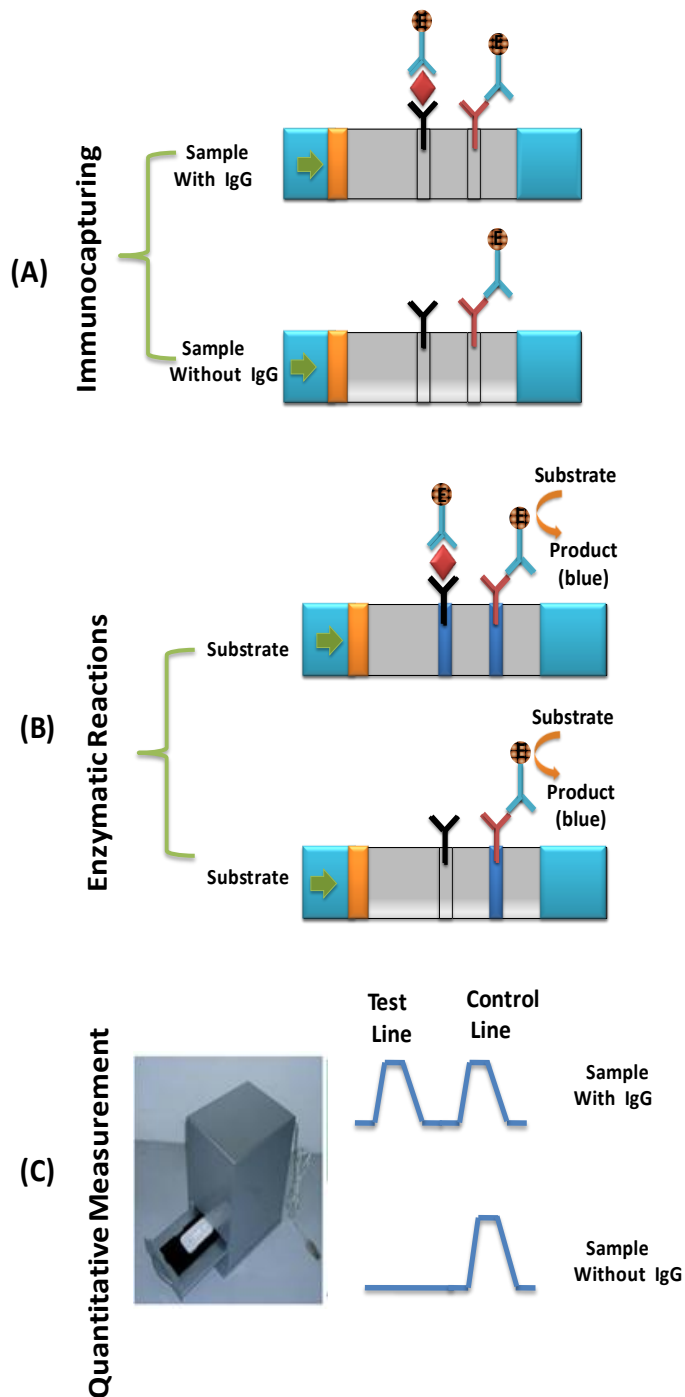

Figure 1. Procedures of immunocapturing (A), enzymatic reactions (B) and quantitative measurement (C) on the enzyme-based dry-reagent strip biosensor.

Then the immunoreactions between IgG and HRP-anti-IgG conjugates occurred and the formed IgG-anti-IgG-HRP complexes continue to migrate along the membrane. When reached the test zone, the complexes were then captured by the poly-anti-IgG immobilized on the test zone via the

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secondary immunoreactions between the poly-anti-IgG and IgG, resulting the sandwich-type complexes, anti-IgG-IgG-anti-IgG-HRP (**Figure 1 (A)**). The capillary action caused liquid sample to migrate further. Once the solution passed through the control zone, the excess HRP-anti-IgG conjugates were captured on the control zone via the binding between the secondary antibody (pre-immobilized on the control zone) and the anti-IgG (**Figure 1 (A)**). After a complete assay (about 7 min), $20\,\mu L$ of the substrate (4-chloro-1-naphthol) solution containing hydrogen peroxide solution was applied. The enzymatic reaction proceeded for 2 min to deposit blue enzymatic insoluble product (4-chloro-1-naphthon) on the test zone and control zone of the DRSB, showing two deep blue lines on the membrane (**Figure 1 (B)**, **top**). In the absence of IgG, only the blue band is observed in the control zone (**Figure 1 (B), bottom**). In this case, the blue band in the control zone (control line) shows that the DRSB is working properly. Qualitative analysis is simply performed by observing the color change of the test zone, and quantitative analysis is realized by reading the optical intensities of the blue bands with a portable strip reader (**Figure 1 (C)**). The peak area is proportional to the amount of the produced enzymatic product in the test zone, which is proportional to the captured enzyme tracers, thus proportional to the concentration of IgG in the sample solution. **Figure 2** presents the typical photo images and corresponding responses of the DRSB in the presence of 0 (a), 50 ng mL^{-1} IgG (b) and 50 ng mL^{-1} IgM (c). There were two visible blue bands appeared only in the presence of IgG (b), and only one band could be seen in the absence of IgG (a) and presence of nonspecific protein IgM (c).

3.2. Optimization of DRSB Fabrication and Assay Parameters

In the current study, HRP was used as a label for tracing antibody-antigen immunoreactions events on the DRSB. The HRP-Ab conjugate was immobilized on the glass fiber by physical adsorption. The intensities of test line and control line depend on the amount of HRP-Ab conjugate captured on the lines, which in turn corresponds to the amount of conjugate in the pad. The amount of

HRP-Ab conjugates on the conjugate pad was controlled by the times of dispensing of the conjugate solution. **Figure 3 (A)** presents the histogram of signal to noise (S/N) ratios of the DRSB with the different dispensing times. It can be seen that the maximum S/N ratio was obtained with the two times dispensing. The further increase of times of dispensing of HRP-Ab conjugates causes an increasingly nonspecific adsorption, resulting a high background and thus low S/N ratio. Therefore the two times dispensing was used to prepare the conjugate pad.
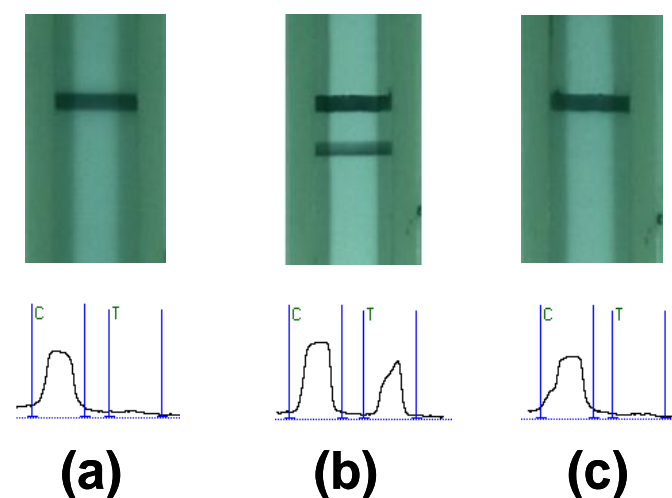

(a) **(b)** **(c)**

Figure 2. Typical photo images and corresponding responses of the enzyme-based DRSB in the presence of 0 (a), 50 ng mL^{-1} IgG (b), and IgM (c). Assay time: 10 min; Sample solution was prepared with 0.01 M PBS 2% BSA; Dispensing times of HRP-anti-IgG conjugate: 2; Concentrations of both primary and secondary antibody for the preparation of the test and control zone are 1.2 mg mL^{-1}. Enzymatic substrate: PBS (0.01 M PBS +15% methanol) containing 8.4 mM 4-chloro-1-naphthol and 4 mM H$_2$O$_2$; Enzymatic reaction time, 2 min.

Another factor affecting the sensitivity and reproducibility of the DRSB is the constituents of the running buffers. Appropriate buffers would minimize the nonspecific adsorption, increase the sensitivity and reproducibility of the sensor. We compared the performances of the DRSB with different running buffers including PBS, PBS +1%BSA and PBS +1% Tween 20, and found that the PBS+1% BSA exhibites the highest S/N ratio (**Figure 3 B**). Furthermore, we found the

concentration of BSA in the running buffer had an effect on the S/N ratio of the DRSB. **Figure 3 (C)** displays the histogram of S/N ratio of the DRSB
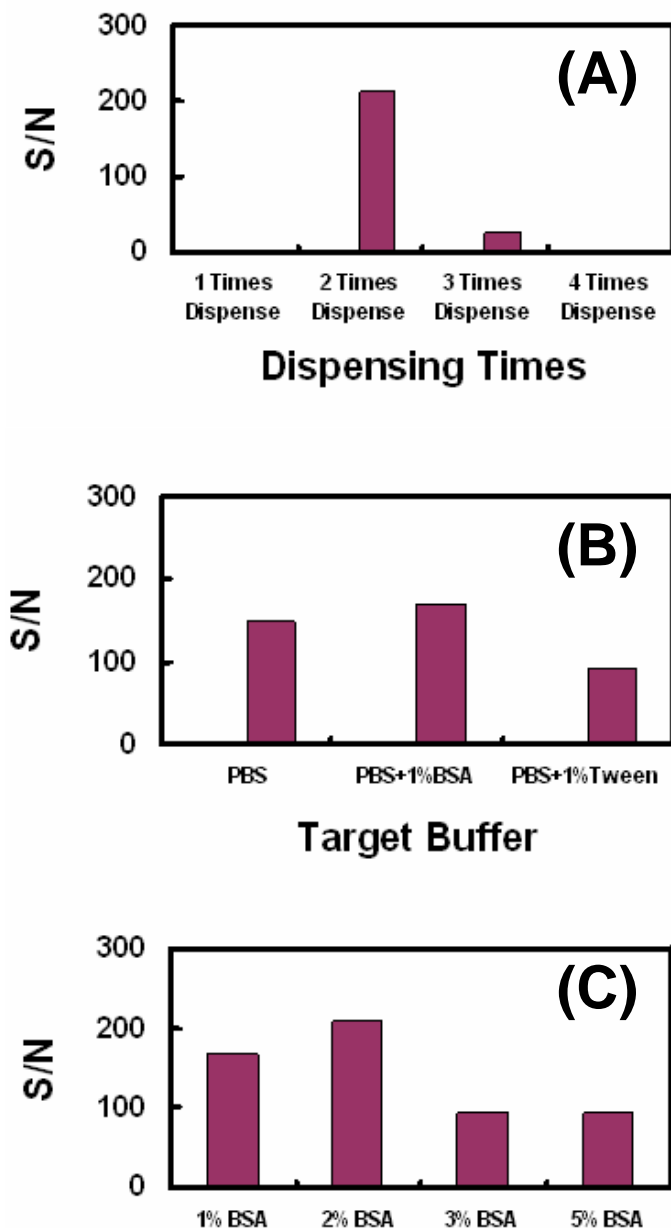

Figure 3. Effect of the times of dispensing of Anti-IgG-peroxidase conjugate (A), the running buffer constituents (B), and the BSA percentage in the running buffer (C) on the response of 111 ng mL^{-1} IgG. Other conditions, as in Fig. 2.

with the PBS buffers containing different concentrations of BSA. One can see that the S/N ratio is increasing with the increase of the BSA concentration (1% to 2%), however any further

increase of the BSA concentration (3% and 5 %) leads to the decrease of S/N ratio. During the optimization, we found the assay time was increasing with the increase of the BSA concentration. It may be caused by the increased viscosity of the buffer due to the increased BSA concentration. Considering the sensitivity of the DRSB and the assay time, a PBS containing 2% BSA was used for the following experiments.

In the current study, the captured HRP tracers on the test and control zones of the DRSB catalyze the substrate 4-chloro-1-naphthol (CN) to produce insoluble blue product (4-chloro-1-naphthon), which deposit on both the test and control zones to form two visualized blue lines. The enzymatic reaction conditions including the concentration of the substrate and enzymatic reaction time would affect the intensities of the lines. **Figure 4 (A)** shows the corresponding plot of the S/N ratio of the DRSB under different enzymatic reaction times. It can be seen that the maximum S/N ratio is obtained with two min enzymatic reaction. Further increase of the enzymatic reaction time increases the background response (control, 0 ng mL^{-1} IgG) and leads to a decreased S/N ratio. So two minutes enzymatic reaction time was used. The effect of substrate concentration on the S/N ratio of the DRSB was also studied by using a sample solution containing 10 ng mL^{-1} R-IgG and two minutes enzymatic reaction time. It was found that the S/N ratio of the DRSB increased up to 8.4 mM CN, then saturate at higher concentrations (**Figure 4 (B)**). So 8.4 mM CN was chosen as a suitable substrate concentration for the current enzymatic reactions.

3.3. Analytical Performances of Enzyme-based DRSB

Under optimal experimental conditions, we examined the performance of the enzyme based DRSB with different concentrations of R-IgG. The quantitative behavior was assessed by monitoring the dependence of the recorded signal of DRSB on the portable strip reader upon the change on the concentration of the R-IgG. Well-defined peaks were observed and the peak area increased linearly with the R-IgG concentration up to 50 ng mL^{-1} and started to level off thereafter (**Figure 5A**). Also shown in inset is a portion of the calibration

plot over a lower concentration range (0-25 ng mL^{-1}). The favorable signal-to-noise characteristics (S/N=3) indicates a detection limit of 0.05 ng mL^{-1} (ppb) from the actual response of 0.1 ng mL^{-1}. The sensitive and specific response was coupled with high reproducibility. A series of six repetitive measurements of 5 ng mL^{-1} R-IgG was used for estimating the precision. This series yielded reproducible signals with a relative standard deviation of 9.7%.
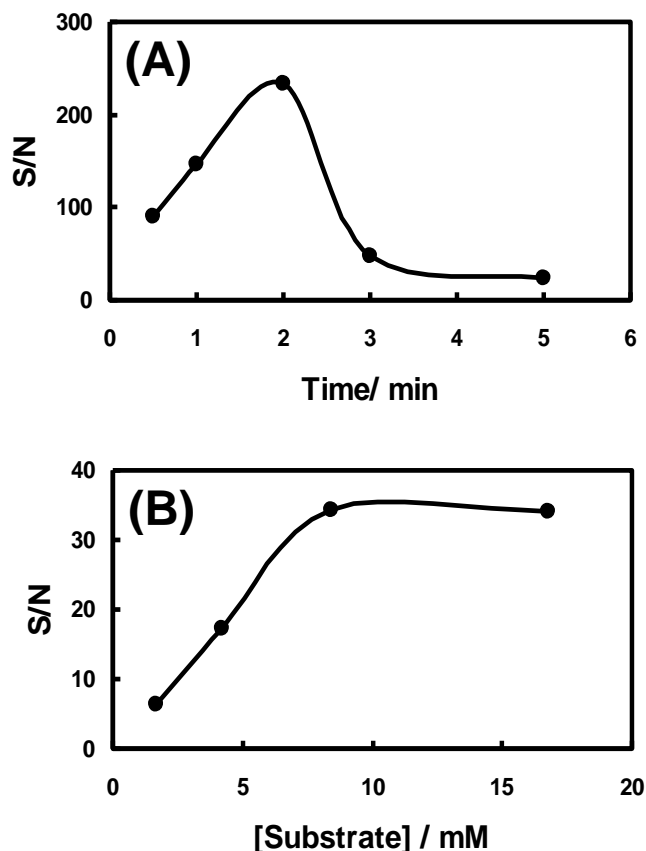

Figure 4. (A) Effect of the enzymatic reaction time on the response of 111 ng mL^{-1} IgG and 8.6 nM enzyme-substrate concentration, and (B) effect of the enzyme-substrate concentration at 2 min enzymatic reaction time on the response of 10 ng mL^{-1} IgG. Other conditions, as in Fig. 2.

3.4. Enzyme-based DRSB vs. Gold Nanoparticle (GNP)-based DRSB

GNP-based DRSB was prepared with the similar preparation procedure of the enzyme-based DRSB. In this case, the GNP labeled anti-IgG was dispensed on the conjugated pad. Figure 6

presents the typical photo images and corresponding optical responses of 50 ng mL^{-1} R-IgG on the two kinds of DRSBs. It can be seen that the visualization of the enzyme-based DRSB test line (**Figure 6 a**) is much better than that of the GNP-based DRSB test line (**Figure 6 b**). The recorded optical response of the test line from the enzyme-based DRSB is three times more than that from the GNP-based DRSB. After optimization, the GNP-based DRSB was capable of detecting 1 ng mL^{-1} R-IgG with a detection limit of 0.5 ng mL^{-1} (results not shown), which is ten times lower than that of the enzyme-based DRSB. However, the reproducibility of both GNP- and enzyme-based DRSB is about the same.
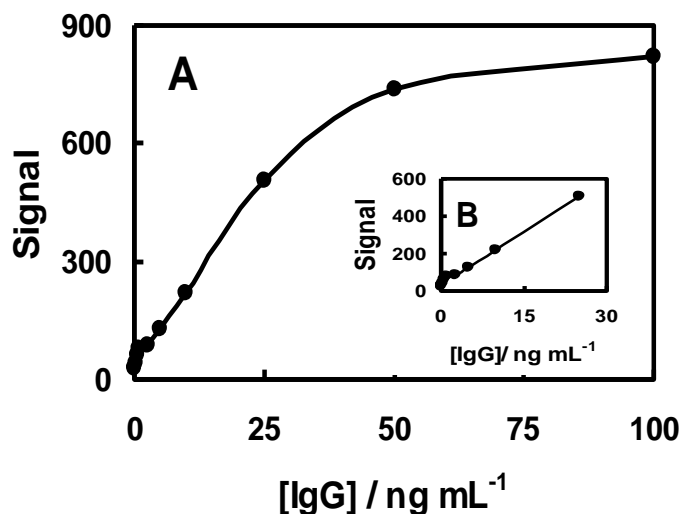

Figure 5. Calibration plot of the enzyme-based DRSB with different concentrations of R-IgG. Inset: First portion of the calibration curve. Other conditions, as in Fig. 2.

3.5. Detecting CEA in Human Plasma with the Enzyme-based DRSB

To evaluate the feasibility of the enzyme-based DRSB, a pair of antibodies including HRP conjugated monoclonal antibody (against CEA) and anti-human CEA polyclonal antibody were used to prepare the enzyme-based DRSB for the detection CEA in human plasma. The plasma samples were prepared by spiking different amount of standard CEA in human plasma. In a typical assay, 25 μL of plasma was applied on the sample pad, and 105 μL running buffer was added

to push the sample through the DRSB. It was found that the recorded peak area of the DRSB was proportional to the logarithm of CEA concentration in the range of 6.5 to 650 ng mL^{-1} (See Figure S2 in Supporting Information) The detection limit of the DRSB was estimated to be 2.5 ng mL^{-1} (S/N=3) within 10 min total assay time. Such detection limit is comparable to that of commercial CEA ELISA kit (0.64 ng mL^{-1}, BIO-QUANT INC, SAN DIEGO, CA).
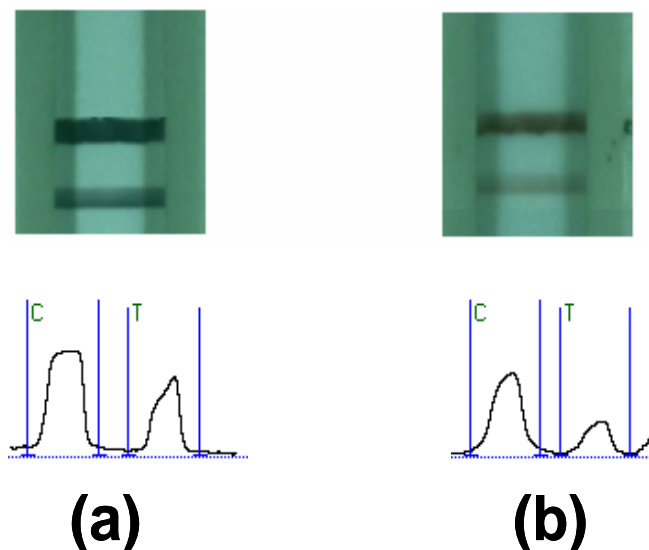

(a)   (b)

Figure 6. Typical photo images and corresponding responses for 50 ng mL^{-1} IgG using the enzyme- (a) and GNP-based (b) DRSB. GNP-based assay time: 15 min; Sample solution was prepared with PBS+2%BSA; Dispensing times of GNP-Ab conjugate: 2; Other conditions, as in Fig. 2.

4.0 Conclusions

We have successfully developed POC DRSB based on enzyme labels and the portable strip reader. The concept was first demonstrated by using an IgG model system. Comparing with the GNP based DRSB, the sensitivity of enzyme based DRSB increased 10 times and it was capable of detecting 0.05 ng mL^{-1} IgG in 10 min. The feasibility of such enzyme based DRSB was evaluated by detecting CEA in human plasma. The detection limit of the enzyme-based DRSB is comparable with that of commercial CEA ELISA

kit. The promising characteristics (fast, sensitive, low-cost and disposable) make the enzyme-based DRSB become a potential substituent for the traditional ELISA kit. Such enzyme-based DRSB can be used for various protein detections and make them particularly attractive for various decentralized and field applications. Current efforts in our laboratory are aimed at developing enzyme-gold nanoparticle dual labels based DRSB (combining the unique optical properties of GNP and enzyme catalytic amplifications) for ultrasensitive protein detection.
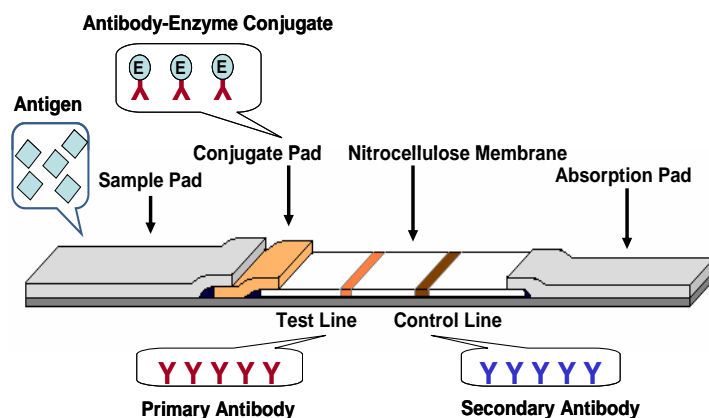
Supporting information

Figure S1. Schematic representation of the point-of-care enzyme-based dry-reagent strip biosensor.
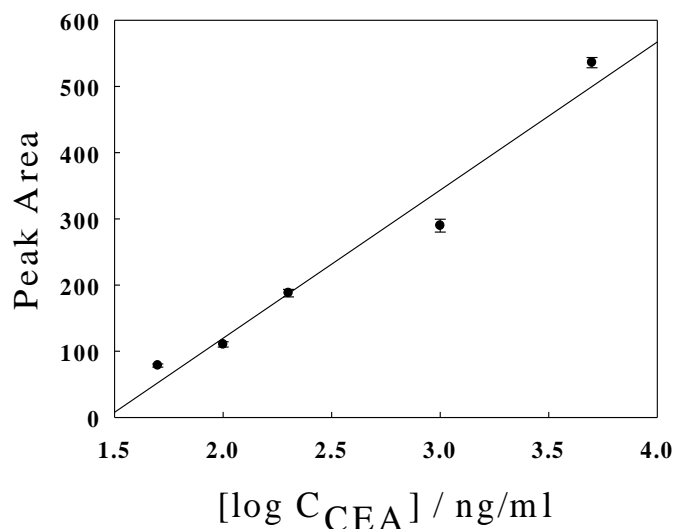
Figure S2 Calibration plot of the enzyme-based DRSB for the detection of CEA in human plasma. Assay time: 10 min. The volume of plasma used: 25 μL. Other conditions, sample as in Figure 2.

References

1. Liu G.; Lin, Y., Nanomaterial labels in electrochemical immunosensors and immunoassays, Talanta, 2007, 74, 308-317.
2. Liu, G.; Wang, J.; Wu, H.; Lin, Y.; Lin, Y., Nanovehicles based bioassay labels, Electroanalysis, 2007, 19, 777-785.
3. Lequin, R.M., Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA), Clinical Chemistry, 2005, 51, 2415-2418.
4. Soper, S.A.; Brown, K.; Ellington, A.; Frazier, B.; Garcia-Manero, G.; Gau, V.; Gutman, S.I.; Hayes, D.F.; Korte, B.; Landers, J.L.; Larson, D.; Ligler, F.; Majumdar, A.; Mascini, M.; Nolte, D.; Rosenzweig, Z.; Wang, J.; Wilson, D., Point-of-care biosensor systems for cancer diagnostics/prognostics, Biosensors and Bioelectronics, 2006, 21, 1932-1942.
5. Durst, R.A; Siebert, S.T; Reeves, S.G., Immunosensor for extra-lab measurements based on liposome amplification and capillary migration, Biosensors and Bioelectronics, 1993, 8, xiii-xv.
6. Lee, K.S.; Kim, T-H.; Shin, M-C.; Lee, W-Y.; Park, J-K., Disposable liposome immunosensor for theophylline combining an immunochromatographic membrane and a thick-film electrode, Analytica Chimica Acta, 1999, 380, 17-26.
7. Weller, M.G., Immunochromatographic techniques - a critical review, Fresenius` Journal of Analytical Chemistry, 2000, 366, 635-645.
8. Jin, Y.; Jang, J-W.; Han, C-H.; Lee, M-H.; Development of ELISA and immunochromatographic assay for the detection of gentamicin, Journal of Agricultural and Food Chemistry, 2005, 53, 7639-7643.
9. Collinson, P.O., The need for a point of care testing: an evidence-based appraisal, Scandinavian journal of clinical and laboratory investigation supplementum, 1999, 230, 67-73.
10. Toshio, W.; Yuichi, O.; Hidekazu, M.; Hiroshi, K.; Yasunori, S.; Yasuhiko, O.; Takao, T.; Yasushi, K., Development of a simple whole blood panel test for detection of human heart-type fatty acid-binding protein, Clinical Biochemistry, 2001, 34, 257-263.
11. Heeschen, C.; Goldmann, B.U.; Langenbrink, L.; Matschuck, G.; Hamm, C.W., Evaluation of a rapid whole blood ELISA for quantification of troponin I in patients with acute chest pain, Clinical Chemistry, 1999, 45, 1789-1796.
12. Liu, G.; Lin, Y-Y; Wang, J.; Wu, H.; Wai, C.M.; Lin, Y., Disposable electrochemical immunosensor diagnosis device based on nanoparticle probe and immunochromatographic strip, Analytical Chemistry, 2007, 79, 7644-7653.
13. Zuk, R.F.; Ginsberg, V.K.; Houts, T.; Rabble, J.; Merrick, H.; Ullman, E.F.; Fischer, M.M.; Slzto, C.C.; Stiso, S.N.; Utman, D.J., Enzyme immunochromatography-quantitative immune-assay requiring no instrumentation, Clinical Chemistry, 1985. 31, 1144-1150.
14. Chen, R.; Li, T.M.; Merrick, H.; Parrish R.F.; Bruno, V.; Kwong, A.; Stiso, C.; Litman D.J., An internal clock reaction used in a one-step enzyme immunochromatographic assay of

theophylline in whole blood., Clinical Chemistry, 1987, 33, 1521-1525.

15. Ono, T.; Kawamura, M.; Arao, S.; Nariuchi, H., A highly sensitive quantitative immunochromatography assay for antigen-specific IgE, Journal of Immunological Methods, 2003, 272, 211 – 218

16. Cho, J.; Paek, E.; Cho, I.; Paek, S., An enzyme immunoanalytical system based on sequential cross-flow chromatography, Analytical Chemistry, 2005, 77, 4091-4097.

17. Cho, J.; Han, S.; Paek, E.; Cho, I.; Paek, S., Plastic ELISA-on-a-chip based on sequential cross-flow chromatography, Analytical Chemistry, 2006, 78, 793-800.

18. Xu, H.; Mao, X.; Zeng, Q.; Wang,S.; Kawde, A., Liu, G., Aptamer-functionalized gold nanoparticles as probes in a dry-reagent strip biosensor for protein analysis, Analytical Chemistry, 2009, 81, 669-675.

19. Mao, X.; Ma, Y.; Zhang, A.; Zhang, L.; Zeng, L.; Liu. G., Disposable nucleic acid biosensors based on gold nanoparticle probes and lateral flow strip, Analytical Chemistry, 2009, 81, pp 1660–1668.

20. Chu, X.; Fu, X.; Chen, K.; Shen, G-L.; Yu, R-Q., An electrochemical stripping metallo-immunoassay based on silver-enhanced gold nanoparticle label, Biosensors and Bioelectronics, 2005, 20, 1805–1812.

21. Mao, X.; Baloda, M.; Gurung, A.S.; Lin, Y.; Liu, G., Multiplex electrochemical immunoassay using gold nanoparticle probes and immunochromatographic strips, Electrochemistry Communications, 2008, 10, 1636-1640.