



Dry-reagent nucleic acid biosensor based on blue dye doped latex beads and lateral flow strip

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ARTICLE INFO

Article history:

Received 5 March 2013

Received in revised form

17 April 2013

Accepted 21 April 2013

Available online 6 May 2013

Keywords:

Lateral flow strip

DNA detection

Latex beads

ABSTRACT

In the manuscript, a quantitative lateral flow nucleic acid biosensor (Lateral flow nucleic acid biosensor, LFNB) based on blue dye doped latex beads was proposed and its feasibility for detecting deoxyribonucleic acid (DNA) in plasma was investigated. A 60-mer DNA sequence (T1) was selected as model to demonstrate the protocol. Blue dyes doped latex bead bearing DNA probe would be captured on the corresponding test line in the presence of target DNA, to form an evident blue band. Although qualitative tests are realized by observing the color change of the test zone, quantitative data are obtained by recording the optical responses of the test zone with a portable "Strip Reader" instrument conveniently. The strip has been applied for the detection of synthesized DNA sample in human plasma sample with a detection limit of 3.75 fmol. Interference was not evident even the target DNA was spiked with 50 μ L plasma which indicated the well shielding of the latex bead reporters and quantified chromatographic separations of unwanted materials of the strip comparing with traditional gold nanoparticle based LFNB platforms.

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1. Introduction

The nucleic acid test is of central importance to the diagnosis and treatment of genetic diseases, to the detection of infectious agents, drug discovery, or warning against bio-warfare agents [1–4]. Developing sensitive and specific DNA detection platforms for use in fundamental research and clinical applications cause extensive attentions in recent years. Several strategies, including real-time PCR, DNA microarrays and surface plasmon resonance [5–7], are widely used for DNA test. While powerful, these approaches are frequently time- and labor-intensive and require expensive instrumentation, which limits their application in laboratory and point-of-care settings.

Lateral flow test strip (LFTS) were first developed for antibody-based pregnancy test in 1990, since then, it was widely applied in detecting of various biomolecules such as protein, enzyme and cells [8]. Comparing with traditional immunoassay technologies, LFTS based immunoassay offer a simple, rapid and cost-effective tool for in-field test. Considering these unique advantages of LFTS, recently, research has concentrated on the developing of POC nucleic acid biosensors based on LFTS technologies.

As we know, the kinetics of nucleic acid hybridization in lateral flow are quite different and more complex as compared with the

formation of the antigen–antibody complex used in common immunochromatography assay, most of reports to detect nucleic acid sequences with LFNA test strips are based on the formation of hapten–antibody or hapten–protein (e.g., biotin–avidin) complexes when the PCR products are amplified using a hapten labeled primer. The other alternative way is based on hybridization reactions of PCR products and specific sequences. These methods have enough sensitivity for amplified DNA detection. For example, Fong et al. [9], Piepenburg et al. [10] and Corstjens et al. [11] use a lateral flow immunoassay for the detection of DNA amplification product. Baeumer et al. reported a dipstick-type LFNA biosensors with nanomolar detection limits based on dye-encapsulating liposome labels [12,13]. The biosensors have been applied to detect Dengue virus in blood samples [14] and viable *Escherichia coli* in drinking water [15]. Ioannou and Christopoulos's group reported a dry-reagent strip biosensor based on oligonucleotide functionalized gold nanoparticles for visual detection of DNA [16]. The biosensors have been used for visual detection of genetically modified organisms, [17] leukemia-related chromosomal translocations, [18] molecular diagnosis of bacterial infection [19] and genotyping of single-nucleotide polymorphisms [20]. In these reported LFNA test strips, the actual hybridization reaction is generally performed before the flow. The pre-hybridization reaction time usually takes 10–30 min. Some tests [11,21] require special and expensive hardware for quantitative detections. We developed some LFTS technologies for DNA tests by using gold nanoparticle as reporter probes for visual test of special DNA

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sequences recently [22,23]. Due to the unique optical characters of gold nanoparticle and enzyme based second amplification protocol, the sensitivity of the LFTS based DNA test tools were greatly improved. Moreover, pre-amplification procedures based on PCR is not necessary and the whole assay time is less than 20 min with detection limits of pM levels.

Considering the relative higher price of gold nanoparticles, some researchers used common dye as reporter probe for proteins detection on LFTS platforms, but the detection limit is pretty high [24,25]. In this study, a quantitative lateral flow nucleic acid biosensor based on blue dyes doped latex bead and lateral flow strip technology (Lateral flow nucleic acid biosensor, LFNB) is proposed and its feasibility for detecting deoxyribonucleic acid (DNA) in plasma is investigated. A pair of DNA probes was designed as capture and reporter probes which were immobilized on nitrocellulose membrane as test line and latex bead, respectively. All reagents used in the test were pre-immobilized and dried on LFNB, so for the test, we just need drop the sample solution on LFNB, waiting for ten more minutes, visual discrimination of the blue band on test line give a “yes” or “no” results. The sensitivity could be further increased by quantitative determinate DNA within several seconds with a portable “Strip Reader” instrument, which is comparable with the performance of our previous reported gold nanoparticle based strip biosensors. The strip biosensor also shows great assay performance for detection of synthesized DNA sample spiked in human plasma sample.

2. Experimental

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). Portable strip reader DT1030 was purchased from Shanghai Goldbio Tech. Co., LTD (Shanghai, China).

2.2. Reagents

Streptavidin from *Streptomyces avidin*, sucrose, Tween 20, dithiothreitol (DTT), Triton X-100, bovine serum albumin (BSA) and sodium chloride-sodium citrate (SSC) Buffer 20 × concentrate (pH 7.0), phosphate buffer saline (PBS, pH 7.4, 0.01 M) were purchased from Sigma-Aldrich. Glass fibers (GF0000800), cellulose fiber sample pads (CFSP001700), laminated cards (HF000MC100) and nitrocellulose membranes (HFB18004 and HFB 24004) were purchased from Millipore (Billerica, MA). Human plasma samples were purchased from Golden West Biologicals (Temecula, CA). DNA oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA) and had the following sequence:

Target DNA: 5'-TTCCCTAGCCCCCAGTGTGCAAGGGCAGTGA-AGACTT

GATTGTACAAAATACGTTTTG-3'

DNA probe 1: 5'-amino-C6-D/CAA AAC GTA TTT TGT ACA AT-3'

DNA probe 2: 5'-ACA CTG GGT GGG CTA GGG AA/Biotin/-3'

DNA probe 3: 5'-Biotin/ATT GTA CAA AAT ACG TTT TG-3'

Noncomplementary DNA: 5'-ATG GCA TCG CTT AGC TGC CAG TAC ACT GAT TGA AGA CAT CAT AGT GCA GAC AAG CAT ATC-3'

All chemicals used in this study were analytical reagent grade. All other solutions were prepared with ultrapure (> 18 MΩ) water from a Millipore Milli-Q water purification system (Billerica, MA).

2.3. Preparation of latex bead-DNA conjugates

Blue dyes doped latex beads (modified with carboxyl group) with average diameter 390 nm were purchased from Signanoprobe company. An oligonucleotide modified with amino group (DNA probe 2) was used for conjugation with latex beads. Before conjugation reaction, the latex beads was activated by the following procedure: 25 μL of 5 mg/mL latex beads was washed with 25 mM MES (pH 6.0) for two times, and dispensed in 500 μL MES buffer containing 25 mg EDC and NHS to react for 30 min. The activated beads were washed three times with 500 μL MES buffer, dispensed in 500 μL MES buffer containing 80 nmol DNA probe 1 to react for 1.5 h. After centrifuge at 8000 rpm for 6 min and discard the supernatant, the DNA modified latex beads were dispensed in 500 μL 50 mM Tris-HCl buffer to react for 15 min to block the active sites of latex beads. Additional three washes with 10 mM pH 7.40 PBS buffer were used to remove the excess DNA probes. Finally, the obtained conjugates were dispensed in a buffer containing 20 mM Na₃PO₄, 5% BSA, 0.25% Tween and 10% sucrose.

2.4. Preparation of lateral flow nucleic acid strip

A schematic diagram of the LFNA test strip is shown in Fig. 1. The LFNA test strip consists of four components: sample application pad, Latex bead-DNA conjugate pad, nitrocellulose membrane and absorbent pad. All of the components were mounted on a common backing layer (typically an inert plastic, e.g., polyester) using the Clamshell Laminator (Biodot, CA, USA). The sample application pad (17 mm × 30 mm) was made from glass fiber (CFSP001700, Millipore) and saturated with a buffer (pH 8.0) containing 0.25% Triton X-100, 0.05 M Tris-HCl and 0.15 M NaCl. Then it was dried and stored in a desiccator at RT. The conjugate pad (8 mm × 30 mm) was prepared by dispensing a desired volume of latex bead-DNA conjugate solution onto the glass fiber pad with the dispenser Airjet AJQ 3000, and then drying it at RT. The pad was stored in a desiccator at 4 °C. Nitrocellulose membrane (25 mm × 30 mm) was used to immobilize the capture DNA probes (DNA probe 1) and control DNA probes (DNA probe 3) at different zones to form test line and control line, respectively. To facilitate its immobilization on the nitrocellulose membrane, streptavidin was used to react with the biotinylated DNA probes to form the streptavidin-biotin DNA conjugates. Briefly, 60 μL of 1 mM biotinylated DNA probes and 140 μL of PBS were added to 300 μL of 1.67 mg/mL streptavidin solution, and the mixture was incubated 1 h at RT. The excess DNA probes were removed by

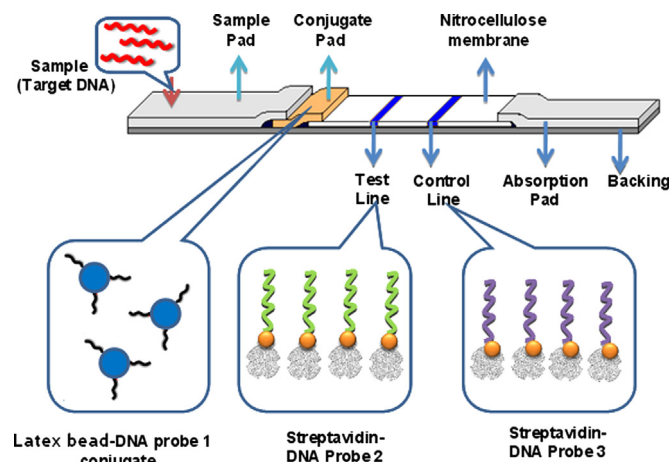


Fig. 1. Components of blue latex beads based lateral flow nucleic acid test strip. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

centrifugation for 20 min with a centrifugal filter (cutoff 30,000, Millipore) at 6000 rpm. The conjugates were washed three times with 1000 μ L of PBS in the same centrifugal filter. Finally, 500 μ L of PBS was added into the remaining solution in the filter. The conjugates were then dispensed on the nitrocellulose membrane with the dispenser Biojet BJQ 3000. The distance between the test line and control line is around 0.2 cm. The DNA probe loaded membrane was then dried at RT for 1 h and stored at 4 °C in a dry state. Finally, The sample pad, conjugate pad, nitrocellulose membrane, absorption pad were assembled on a plastic adhesive backing (60 mm $\times$ 30 mm) using the clamshell laminator. Each part overlaps 2 mm to ensure the solution migrating through the strip during the assay. Strips with a 4 mm width were cut by using the Guillotin cutting module CM 4000.

2.5. Sample assay procedure

For the optimization experiments, the procedure of FLNA test is following: One hundred thirty microliters of sample solution containing a desired concentration of target DNA in 4 $\times$ SSC (10% Tween-20) buffer was applied to the sample application zone. After waiting for a desired time (for example, 10 min), another 50 μ L 4 $\times$ SSC (10% Tween-20) buffer was applied to wash the strip. The bands were visualized within 15 min. For quantitative measurements, the strip was inserted into the strip reader DT1030, the optical intensity of the test line and control line could be recorded simultaneously by using the “GoldBio strip reader” software.

In the case of the detection of DNA in plasma sample, 50 μ L of human plasma spiked with certain concentration of DNA was first applied to the strip, and then 70 μ L of 4 $\times$ SSC (10% Tween-20) buffer was added to the sample pad to push the plasma pass through the strip. After 15 min, the strip was read with the strip reader.

3. Results and discussion

3.1. Principle of lateral flow nucleic acid test strip

The principle of the LFNA test strip is based on an on-strip sandwich DNA hybridization reaction and the protocol is illustrated in Fig. 1. In this study, three DNA probes were used: DNA probe 2 and DNA probe 3 were immobilized on the nitrocellulose membrane to form the test line and control line, respectively. DNA probe 1 was attached to the latex beads and dispensed in the conjugate pad (glass fiber). The sample solution containing target DNA is applied on the sample application pad. The solution migrates by capillary action and passes the conjugate pad, and then rehydrates the latex bead-DNA probe 1 conjugates. The target DNA hybridizes with DNA probe on the latex bead to form the complex and continue to migrate along the strip. The hybrids are captured on the test line by the second hybridization between the target DNA and the immobilized DNA probe 2. The accumulation of latex beads in the test line of the nitrocellulose membrane is visualized as a characteristic blue band. The excess of latex bead-DNA probe 1 conjugates continue to migrate and pass the control line, in which the DNA probe 3 is immobilized. Then the excess of latex bead-DNA probe 1 are captured by the hybridization between the DNA probe 1 and the DNA probe 3, thus forming a second blue band. In the absence of target DNA, no blue band is observed in the test line. In this case, a blue control band shows that the LFNA test strip is working properly. Qualitative analysis is simply performed by observing the color change of the test line, and quantitative analysis is realized by reading the optical intensity of the test line with a portable strip reader.

Fig. 2 presents the typical photo images and corresponding optical responses of 0 nM target DNA (control, a), 25 nM noncomplementary

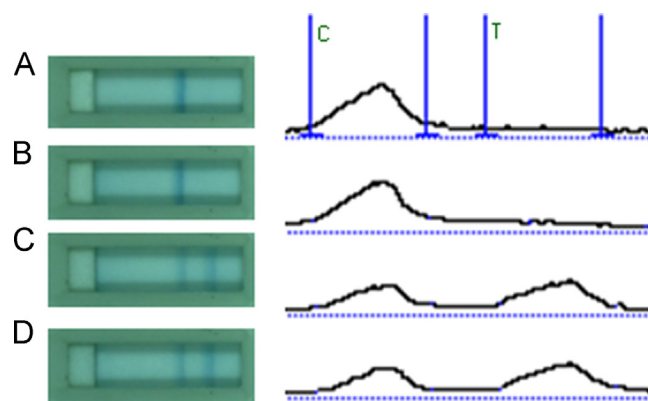


Fig. 2. Typical photo images and recorded responses of the blue latex beads-based strip biosensor in the presence of (A) running buffer (4X SSC, 10% Tween-20); (B) 25 nM noncomplementary DNA; (C) 2.5 nM target DNA; (D) 2.5 nM target DNA and 25 nM noncomplementary DNA. Assay time, 15 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DNA (b) and 2.5 nM target DNA (c) and the mixture of 25 nM noncomplementary DNA and 2.5 nM target DNA (d). Two blue bands were observed in the presence of target DNA, and only one blue band (control line, top) was observed in the absence of target DNA and presence of excess noncomplementary DNA. The presence of excess noncomplementary DNA does not affect the signal of target DNA (d). The intensities of the bands were recorded by the strip reader shown on the right of the figure. Well-defined peaks were observed and the peak areas were recorded according to the captured latex beads in the test line (right side) and control line (left side).

3.2. Optimization of strip fabrication and assay Parameters

The most important factor to affect the sensitivity and reproducibility of the strip test is the use of various buffers. Appropriate buffers would minimize the nonspecific adsorption, increase the sensitivity and reproducibility of the strip test. In the current study, four kinds of buffers were used in the strip fabrication and strip test: (1) The buffer (pH 8.0) containing 0.25% Triton X-100, 0.05 M Tris-HCl and 0.15 M NaCl was used to saturate the sample pad. This treatment would facilitate to transport the target DNA into a downstream portion of the lateral flow device and reduce entrapment of target DNA in the sample pad. (2) During the preparation of latex bead-DNA conjugates, the latex bead-DNA pellets were dispersed in the buffer containing 20 mM Na_3PO_4 , 5% BSA, 0.25% Tween and 10% sucrose. The addition of BSA, Tween 20 and sucrose is to stabilize the latex bead conjugates and facilitate the release of the conjugates from the conjugate pad. Another important function of these buffer components is to reduce the nonspecific adsorption of latex bead-DNA on the nitrocellulose membrane. After rehydrating the conjugates, the components (BSA, Tween 20 and sucrose) are dispersed in the running buffer and migrating along the strip, and will block the nitrocellulose membrane naturally without additional block steps. (3) A 0.01 M PBS buffer (pH 7.4) was used to prepare streptavidin-biotinylated DNA probe solutions, which were used to prepare the test line and control lines. (4) The most important target of the optimization of the strip's performance is to develop the signal to noise ratio. The similar protocol was tried based on gold nanoparticle previously in our lab. However, the sensitivity of the biosensor was not satisfied, and the bearing ability of the gold nanoparticle labels to plasma was not good (The signal would decrease evidently in the presence of 10 μ L plasma), which greatly limited its real applications. The signal to noise ratio is very low while we first check the possibility of the protocol because the latex beads modified with DNA probes

seems difficult to pass through the nitrocellulose membrane. Experiments indicated that the buffer used for target dilution would affect the flow ratio of the latex beads labels greatly. First, several buffer such as PBS, PBST, Tris-HCl, 4xSSC (1% BSA) and 4xSSC (0.15% Tween-20) were checked in details. However, most of these buffers did not rehydrate the latex bead labels except PBST and 4xSSC (0.15% Tween-20). It seems that tween-20 be good for improving the movement of latex bead labels on the nitrocellulose membrane. Therefore, effect of 4x SSC buffer containing different amount of Tween-20 to the performance of the biosensor were studied. One can see in Fig. 3, the responses obtained would increase with the increasing amount of Tween-20 in the 4x SSC buffer. For the sample solution containing Tween-20 less than 15% (0.10, 0.15, 0.50, 5 and 10%), the difference of assay time is not evident (data not shown). But their responses would decrease with decreasing of Tween-20 concentration, because less Tween-20 in the sample solution leads to less releasing of latex bead labels, so lower responses would be observed. While the Tween-20 concentration is up to 10%, most latex bead labels could be released completely and the assay time is about 15 min. Higher optical response would be obtained for 4x SSC (15% Tween-20) buffer, however, the flow rate of the latex bead labels would become slowly which delay the assay time (Due to the elevating of solution viscosity. Typically, the whole assay time would increase to 25 min). Considering the balance of detection sensitivity and time, 4x SSC (10% tween-20) was used for following experiments.

Secondary, the amount of DNA probe on the latex beads surface would affect the response of the biosensor. Theoretically, one DNA probe per latex beads would give the maximum sensitivity, because one target DNA would bind with one latex bead. Considering the short hybridization time on the strip test, multiple probes attached latex beads may increase the hybridization efficiency and sensitivity. We studied the effect of DNA probe quantity during the conjugate process on the signal of the strip (Fig. 4). The response signal (peak area) increases upon raising the DNA amount in the conjugate solution from 20 to 80 nmol. High quantity of DNA probes was not checked considering the consuming of reagent and the cost per assay. A DNA probe amount of 80 nmol in the conjugate solution was used to prepare the latex beads-DNA conjugates for the following experiments.

In the current study, the latex beads-DNA conjugate was immobilized on the glass fiber by physical adsorption, namely, the conjugate pad. The intensities of test line and control line depend on the amount of latex beads-DNA conjugate captured on the lines, which in turn corresponds to the amount of conjugate in the conjugate pad. The amount of latex beads-DNA conjugates on the conjugate pad was controlled by the dispensing volume of the conjugate solution. Bio-dot system (Millipore corporation, USA) was used to dispense DNA probe conjugated beads on the conjugate pad. For increasing the amount of DNA probe

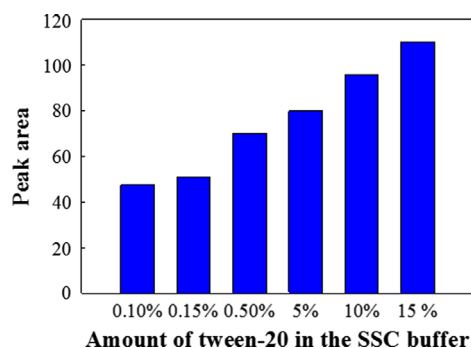


Fig. 3. Effect of concentration of tween-20 in the running buffer on the response of 2.5 nM target DNA. Assay time: 15 min.

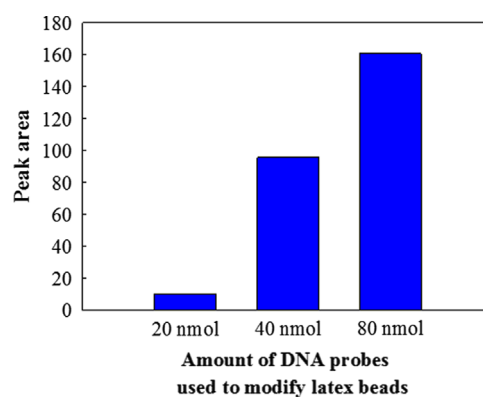


Fig. 4. Effect of amount of DNA probes used to modify blue latex beads. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

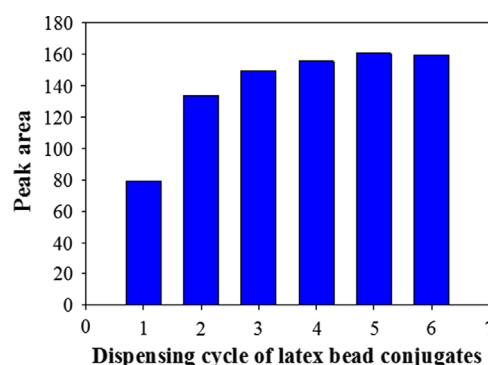


Fig. 5. Effect of dispensing cycle of blue latex bead-DNA conjugates on conjugate pad. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conjugated bead, we dispense DNA probe conjugated bead on the conjugate pad for many times. The concentration of DNA probe conjugated bead and dispensing volume were set before dispensing, so the amount of the conjugates is precise for individual lateral flow strip biosensor if certain dispensing cycles were applied on the conjugate pad. Fig. 5 presents the bar-graph of the recorded intensity of test line for 2 nM of target DNA test. The responses of the strip test increased up to 5 dispensing cycles on the conjugate pad and then the response signal tends to be stable. It is easy to imagine that more conjugated beads would be located on conjugate pad with further increasing dispensing cycles of the conjugated bead. So the releasing time of the conjugated bead would definitely increase, which lead to longer assay time in turn. So 5 dispensing cycles of the conjugates was used as the optimal cycles for most experiments considering the balance of sensitivity and assay time.

3.3. Analytical performances

To investigate whether the biosensor could provide quantitative detection of target DNA, the intensities of the test zones were estimated and plotted as a function of different concentrations of target sequence (Fig. 6A). It was observed that well-defined peaks occurred, and the peak areas increased with an increase in the target DNA concentration (Fig. 6B). The useful analytical range extended from 0.25 to 50 nM of complementary DNA and was suitable for quantitative work (Fig. 6C). Since quantitative analysis relies on the stability of the analyte signal and reproducibility of the assay, and to determine the reproducibility of the biosensor,

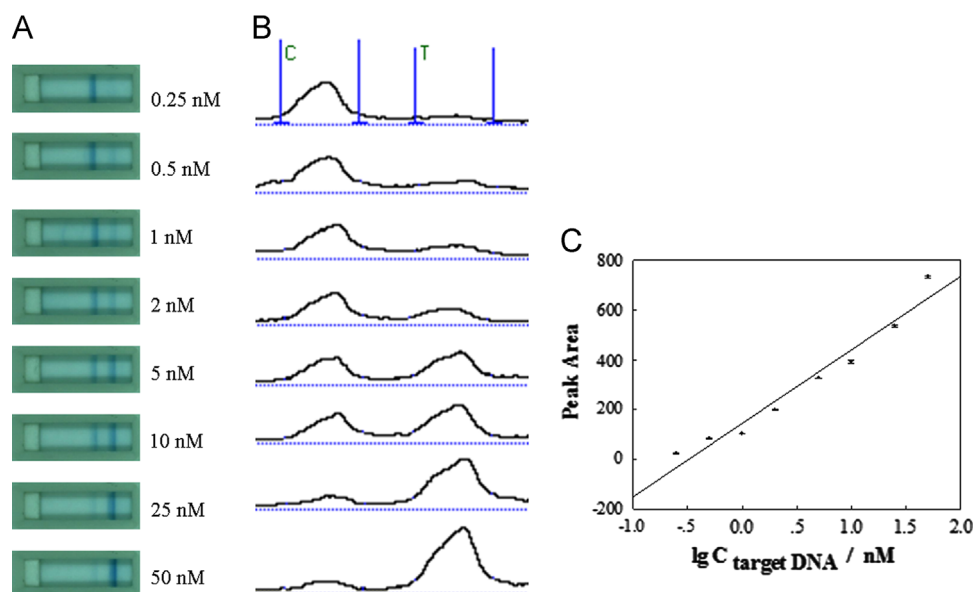


Fig. 6. Typical photo-images (A) and corresponding responses (B) of the blue latex bead based strip biosensor in the presence of different concentration of complementary target DNA, and the Calibration curve for detection of target DNA (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

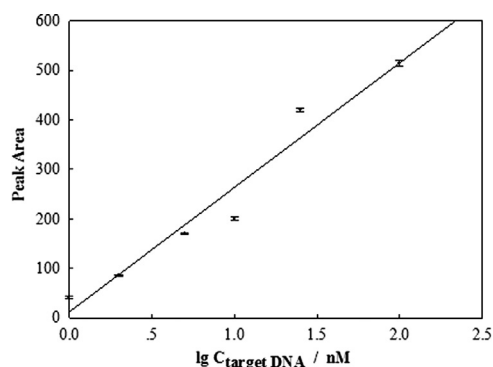


Fig. 7. Calibration curve for target DNA detection in plasma.

samples of 2.5 nM complementary DNA were loaded on six different biosensors that gave reproducible signals with a relative standard deviation (RSD) of 8.3%. The detection limit was 100 pM (based on $S/N=3$) in connection with a 15 min assay time.

To test the practicality of the biosensor, experiments were performed in a complex sample matrix, such as plasma. The sample solutions were prepared by spiking different quantities of target DNA solution into the plasma. The addition of plasma would lead to the matrix effect, so the volume of plasma was optimized before the experiment. Different volumes of plasma (10–50 μ L) spiked with target DNA was added to the sample application pad, and then the biosensor was washed with running buffer. The signal was recorded with the strip reader after 15 min. A better performance in terms of higher signal-to-noise ratio was obtained even when the volume of plasma was up to 50 μ L, so 50 μ L of plasma was applied in the following experiment. To our previous experiences, it is difficult for gold nanoparticle based strip biosensors to detect biomolecules in large volume of plasma sample due to the unavoidable interference of plasma matrix. The background will increase rapidly as increasing of volume of plasma sample applied on the biosensor. The resulting plot of the peak area versus complementary DNA quantity is linear over the 1.0–100 nM range with a detection limit of 3.75 fmol (based on $S/N=3$, Fig. 7) in 50 μ L plasma.

4. Conclusions

We have developed a dry-reagent strip biosensor based on DNA probe functionalized blue dye doped latex beads for target DNA analysis. A model system comprising a target DNA analyte and a pair of DNA probes was used to demonstrate the proof-of-concept. Under optimal conditions, a linear relationship between the peak area and the target DNA concentration was observed in the range of 0.25–50 nM with a detection limit of 100 pM. The proposed biosensor is capable of detecting target DNA in 50 μ L human plasma with a detection limit of 3.75 fmol, which is not possible for gold nanoparticle labels (due to the serious interference of plasma). Moreover, the presence of excess noncomplementary DNA showed no effect on the biosensor response, illustrating the good selectivity. The success of the blue dye doped latex beads based strip biosensor show superior assay performance for DNA detection in terms of sensitivity and specificity, further works will focus on developing protein detection platforms for in-field test by using antibody pairs.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant no. 21205094) and New Faculty Startup Funds of Northwest University in Shaanxi Province (Grant no. PR12011).

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