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Carbon nanotube-based lateral flow biosensor for sensitive and rapid detection of DNA sequence

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ABSTRACT

In this article, we describe a carbon nanotube (CNT)-based lateral flow biosensor (LFB) for rapid and sensitive detection of DNA sequence. Amine-modified DNA detection probe was covalently immobilized on the shortened multi-walled carbon nanotubes (MWCNTs) via diimide-activated amidation between the carboxyl groups on the CNT surface and amine groups on the detection DNA probes. Sandwich-type DNA hybridization reactions were performed on the LFB and the captured MWCNTs on test zone and control zone of LFB produced the characteristic black bands, enabling visual detection of DNA sequences. Combining the advantages of lateral flow chromatographic separation with unique physical properties of MWCNT (large surface area), the optimized LFB was capable of detecting of 0.1 nM target DNA without instrumentation. Quantitative detection could be realized by recording the intensity of the test line with the Image J software, and the detection limit of 40 pM was obtained. This detection limit is 12.5 times lower than that of gold nanoparticle (GNP)-based LFB (0.5 nM, Mao et al. Anal. Chem. 2009, 81, 1660–1668). Another important feature is that the preparation of MWCNT–DNA conjugates was robust and the use of MWCNT labels avoided the aggregation of conjugates and tedious preparation time, which were often met in the traditional GNP-based nucleic acid LFB. The applications of MWCNT-based LFB can be extended to visually detect protein biomarkers using MWCNT–antibody conjugates. The MWCNT-based LFB thus open a new door to prepare a new generation of LFB, and shows great promise for in-field and point-of-care diagnosis of genetic diseases and for the detection of infectious agents.

1. Introduction

DNA detection has been a topic of central importance in many different fields such as clinical diagnosis, genetics therapy, food safety and warning against biowarfare agents (Cheng et al., 2009; Franca et al., 2002; Klepárník and Bocek, 2007; Riccardi et al., 2007; Nakajima et al., 2013; Obliosca et al., 2013; Sassolas et al., 2008). The most commonly used method for DNA detection is based on the polymerase chain reaction (PCR) due to its exponential amplification capability (Joshi and Deshpande, 2011; Mullis and Faloona, 1987; Rahman et al., 2013). However, what paradox is that even a tiny amount of the contamination can result in enormous false positive. Moreover, the PCR-based detection strategy requires a relatively clean environment, sophisticate and expensive instruments, and long assay time, which make it inconvenience for rapid on-site test. Therefore, great efforts have been made to explore DNA biosensors over the past decade (He et al., 2011; Komarova et al., 2005; Zhang et al., 2013).

Numerous DNA biosensors in connection with different transducers (electrochemical, optical, acoustic, piezoelectric, etc.,) have been reported in the literatures (Baeumner et al., 2004; Mao et al., 2009; Sassolas et al., 2008). Recently the emergence of nanotechnology is opening new horizons for the application of nanomaterials (nanoparticles, nanowire and nanotubes) for sensitive detection of DNA (Mao and Liu, 2008; Liu et al., 2009; Pumeraa et al., 2007; Xu et al., 2009). Even copies of DNA or RNA can be detected without PCR amplification (Cannon et al., 2012; Liu et al., 2012; Nam et al., 2004; Zhang et al., 2004; Zhao et al., 2003). However the applications of DNA biosensors have been limited because of complex operations, high cost, and requirement of trained personnel (He et al., 2011). Recently, lateral flow biosensors (LFB) have attracted considerable attention due to its rapid, portable and low-cost features (Roskos et al., 2013; Terao et al., 2013). Our group and others have successfully devised the lateral flow nucleic acid

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biosensors, which enable visual detection of DNA fragments without instrumentation (Baeumner et al., 2004; Kalogianni et al., 2007; Mao et al., 2009; He et al., 2011, Rastogi et al., 2012). Gold nanoparticles (GNPs) were usually used as labels for the generation of visible signals. However, due to the limited surface area of the GNPs, LFB often suffers from low sensitivity (Zhang et al., 2011). Another issue from the GNP-based LFB is the aggregation of GNP–DNA conjugates during the preparation and test. Since their discovery by Iijima (1991), carbon nanotubes (CNTs) have attracted significant attention because of their unique physical, chemical and electrical properties (Baughman et al., 2002; Kadam, 2009; Park et al., 2013; Qu et al., 2008; Terrones, 2003). Characteristics of CNT such as increased surface area along with enhanced electrical/optical properties make them suitable for numerous applications such as nanoelectronics, photovoltaics and chemical/biological sensing (Kadam, 2009; Park et al., 2013). Due to its large surface area, CNTs have been used as carrier to load large number of enzyme molecules for ultrasensitive DNA detection (Munge et al., 2005; Zhang et al., 2011). However the CNT-based DNA biosensors and bioassays still suffered from tedious assay time, multiple washing steps and the requirement of trained personnel.

In this work, we present a carbon nanotube-based lateral flow biosensor (LFB) for rapid detection of DNA sequence with high sensitivity and short assay time. Amine-modified DNA detection probe was immobilized on the shortened MWCNTs surface via diimide-activated amidation between the carboxylic acid groups on the CNT and amino groups on the detection DNA. The MWCNT–DNA conjugates were used to construct the LFB. Sandwich-type DNA hybridization reactions were performed on the LFB, and the captured MWCNTs on test zone and control zone of LFB produced the characteristic black bands, enabling visual detection of nucleic acid samples without instrumentation. The promising properties of the CNT-based LFB are reported in the following sections.

2. Materials and methods

2.1. Apparatus

The Biojet BJQ 3000 dispenser, Clamshell Laminator, and the Guillotine cutting module CM 4000 were purchased from Biodot LTD (Irvine, CA), Nikon COOLPIX S4200 camera (Nikon, Japan) was used to take the photo images of lateral flow biosensors. A Hitachi HT7700 field transmission electron microscope (TEM; Tokyo, Japan) was used to take images of unshorten and shortened carbon nanotubes.

2.2. Reagents

Carboxylated multi-walled carbon nanotubes (MWCNTs, purity $>95\%$), carboxylated single-walled carbon nanotubes (SWCNTs) (purity $>95\%$), streptavidin, bovine serum albumin (BSA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), 2-(4-Morpholino) ethanesulfonic acid (MES), sucrose, Tween 20, phosphate buffer saline (0.01 M PBS, pH 7.4), and sodium chloride-sodium citrate (SSC) buffer 20× concentrate (pH 7.0) were purchased from Sigma-Aldrich (St. Louis, MO). Cellulose fiber sample pads (CFSP001700), glass fibers (GFCP000800), laminated cards (HF000MC100), and nitrocellulose membranes (HFB18004 and HFB24004) were purchased from Millipore (Billerica, MA).

All the chemicals used in this study were analytical reagent grade. Solutions were prepared with ultrapure ($\geq 18\ M\Omega$) water from Millipore Milli-Q water purification system (Billerica, MA). All the DNA oligonucleotides used in this study were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and had the following sequence:

Target DNA: 5′-ATG ACC TAT GAA TTG ACA GAC-3′.
Amine-modified detection DNA probe: 5′-Amino-GTC TGT CAA-3′.
Biotinylated capture DNA probe: 5′-ATA GGT CAT/Biotin-3′.
Biotinylated control DNA probe: 5′-Biotin/MC6-D/TTG ACA GAC-3′.
Non-complementary DNA 1: 5′-TAA AAA AGG GAG TAA CCG AAA ACG-3′.
Non-complementary DNA 2: 5′-CAT TCC AGC AGC TGT TT-3′.
Non-complementary DNA 3: 5′-CGT TGA ATC CAG CAG AAA AA-3′.
One base mismatched DNA: 5′-ATG ACC TAT GAA TTG AGA GAC-3′.

2.3. Preparation of CNT–DNA conjugates

The carboxylated MWCNTs (or SWCNTs) were firstly treated with mixed concentrated acids (HNO_3:H_2SO_4=1:3) under ultrasonication for 3 h, and washed with water for three times. Then the shortened carboxylated MWCNTs (or SWCNT) (0.5 mg) was mixed with 9.6 mg EDC and 5.43 mg sulfo-NHS in 1.0 mL MES buffer (0.1 M, pH 4.7).After shaking at room temperature for 15 min, the mixture was washed by centrifuging at 10,000 rpm for 5 min. Discard the supernatant and resuspend the pellet in PBS buffer. Repeat the above operations/treatments three times to remove the excess reagents. The amine-modified DNA detection probe was then added to the activated MWCNT (or SWCNT) solution with a final concentration at 0.1 OD mL^{-1} and the solution was incubated overnight at room temperature. This mixture was centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in PBST. After repeating the above step 3 times, the pellet was resuspended in 1 mL eluent buffer containing 20 mM $Na_3PO_4 \cdot 12H_2O$, 5% BSA, 10% sucrose, and 0.25% Tween-20. The CNT–DNA conjugate solution was stored at 4 °C before further use.

2.4. Preparation of streptavidin-biotinylated DNA conjugates

Two hundred microliter of 2.5 mg mL^{-1} of streptavidin was mixed with 50 nmol biotinylated DNA probes (capture DNA probe or control DNA probe). The mixture was incubated on shaker for 1 h. After adding 500 μL PBS into the mixture, the solution was centrifuged with centrifugal filter for 20 min at 6000 rpm at 4 °C. The above step was repeated for 3 times. The remaining solution in filter was diluted to 600 μL with PBS.

2.5. Preparation of the lateral flow biosensor (LFB)

The LFB consists of the following components: sample application pad, conjugate pad, nitrocellulose membrane, and absorbent pad. The sample application pad (17 mm × 30 cm) was made from cellulose fiber (CFSP001700, Millipore) and soaked into sample pad buffer containing 0.05 M Tris–HCl, 0.25% Triton X-100, 2.5% Tween-20 and 0.15 m M NaCl (pH 8.0). Then it was dried at 37 °C for 3 h and stored in desiccators at room temperature. The conjugate pad (8 mm × 30 cm) was prepared by dispensing an optimal volume of MWCNT–DNA conjugate solution onto the glass fiber pad, then it was dried at room temperature and stored at 4 °C. The test and control zones on the nitrocellulose membrane (25 mm × 30 cm) were dispensed by Biojet BJQ 3000 dispenser at a desired volume of streptavidin-biotinylated capture probe (test zone) and streptavidin-biotinylated control probe (control zone)

solutions respectively. The distance between the test and control zones was about 3 mm, and then the membrane was dried at 37 °C for 1 h and stored at 4 °C. Finally, all of the four components were assembled on a plastic adhesive backing (60 mm × 30 cm) using the clamshell laminator. Each part overlapped 2 mm to ensure that the solution could migrate through the strip during the assay. Strips with 3 mm width were cut by the Guillotin cutting module CM 4000.

2.6. Assay procedure

One hundred microliter of sample solution containing different concentration of target DNA in running buffer (1/4 SSC+2% BSA) was added onto the sample pad, then the solution migrated toward absorption pad. The test and control zones could be evaluated visually within 20 min. For quantitative measurements, the optical intensities of the test line and control line was read using Imagine J software. The digital images of the LFB were obtained with Nikon COOLPIX S4200 camera (Nikon, Japan) and then transferred to a computer. After opened in Image J software, the images were converted to 32-bit formats using Image/Type 32-bit command and exaggerated the contrast with Process/Enhance contrast command. In the image window, the test line was amplified using the magnifying glass and outlined with the rectangular selection tool, then the signal was gotten using the Analyze/Measure command. The signal difference was obtained by dragging the rectangular selection to blank position and using the measure command again.

3. Results and discussion

3.1. Preparations of shortened-MWCNTs and MWCNT–DNA conjugates

In current study, carboxylated multi-wall carbon nanotubes (MWCNTs) were used as a label to conjugate with the amine-modified DNA detection probe. MWCNTs play a dual significant role in both the DNA probe immobilization and colored reagent, namely as carriers for DNA immobilization to provide a large surface area to increase the amount of immobilized DNA and hybridization efficiency, and as a colored regent for visual detection of DNA sequences. Considering the length (5–20 μm, Fig. 1A) and the low solubility of untreated MWCNT in water, MWCNTs were shortened with mixed concentrated acids (HNO_3:H_2SO_4 =1:3) under ultrasonication. Fig. 1B presents the typical TEM images of the shortened MWCNTs. One can see that the length of the shortened MWCNT is around 0.5–2 μm. The shortened MWCNT was then used to immobilize the amine-modified detection DNA probe via diimide-activated amidation between the carboxylic acid groups on the CNT and amine groups on the detection DNA probe.

3.2. Principle of LFB measurement

Fig. 2 illustrates the principle of DNA measurement on the MWCNT-based LFB. A target DNA and a pair of DNA probes, which are complementary with the target DNA in two different locations, were used to demonstrate the proof-of-concept. Typically, the sample solution containing target DNA was applied to the sample pad. Subsequently, the solution migrated by capillary action, then the hybridization reactions between target DNA and the detection DNA probe of MWCNTs–DNA conjugates occurred, and the formed complexes (MWCNTs–DNA–target DNA) continued to migrate along the strip. After reaching the test zone, the complexes were captured by the biotinylated capture DNA probe immobilized on the test zone via the second hybridization reaction. A characteristic black band could be observed because of the accumulation of MWCNTs on the test zone. Once the solution passed through the control zone, the excess MWCNTs–DNA conjugates were captured by the biotinylated control DNA probe, thus a second black band appeared. In the absence of target DNA, only the black band in the control zone is observed. In this case, the black band in the control zone (control line) shows that the LFB works properly. Qualitative analysis is simply performed by observing the color change of the test zone, and quantitative analysis is realized by analysis the photo images of the LFB with Image J software.

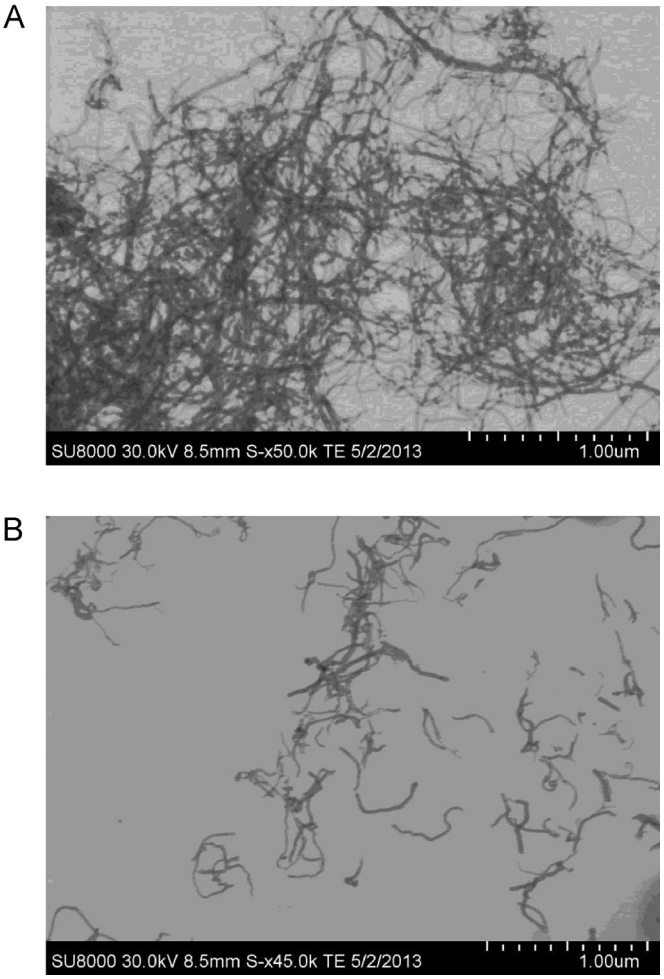

Fig. 1. TEM images of multi-walled carbon nanotubes (A) and the shortened multi-walled carbon nanotubes (B).

Fig. 3A presents the typical photo images of LFBs after measuring the sample solutions containing 0 nM DNA, 1 nM target DNA and 100 nM noncomplementary DNA. There was no test band observed with the 0 nM DNA and the 100 nM noncomplementary DNA while a distinct, black band appeared in the presence of 1.0 nM DNA. The results indicated that the proposed MWCNT-based LFB would detect DNA selectively. We compared the performances of MWCNT- and SWCNT-based LFBs and results are shown in Fig. 3B. It can be seen that the *S*/*N* ratio of MWCNT-based LFB is almost 5 times higher than that of SWCNT-based LFB. Such *S*/*N* ratio difference may be caused by the difference of surface area between MWCNT and SWCNT. The larger surface area would immobilize more DNA probe, and thus offer higher DNA probe density on the CNT surface and higher hybridization efficiency during the test. So we choose MWCNT as a label to optimize the experimental conditions of LFB.

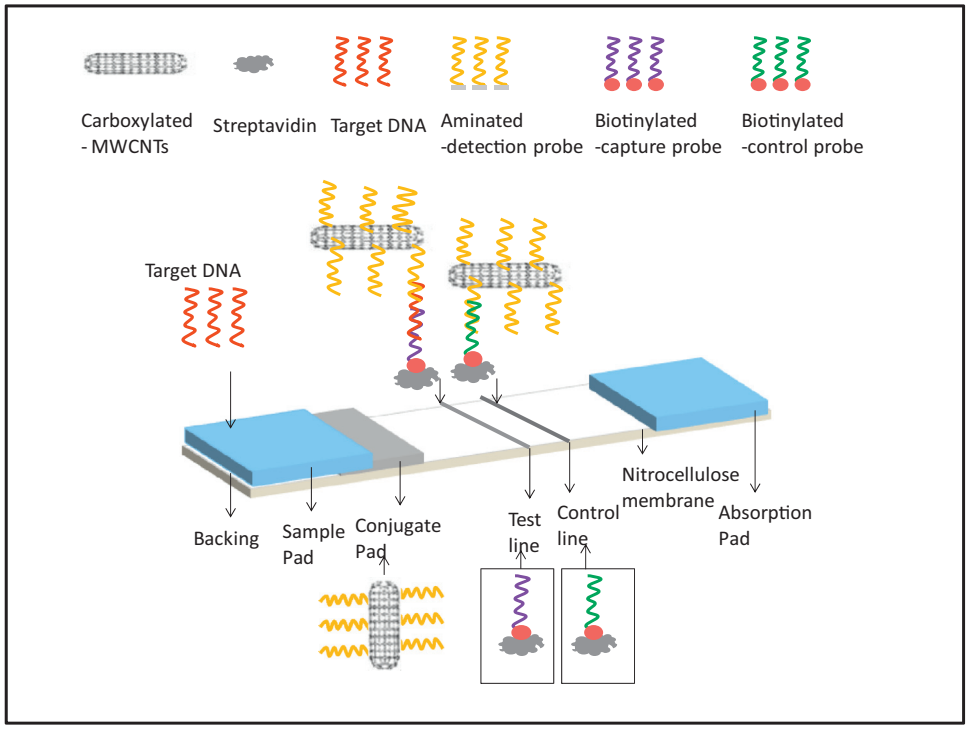

Fig. 2. Schematic illustration of the principle of DNA measurement on MWCNT-based lateral flow biosensor.

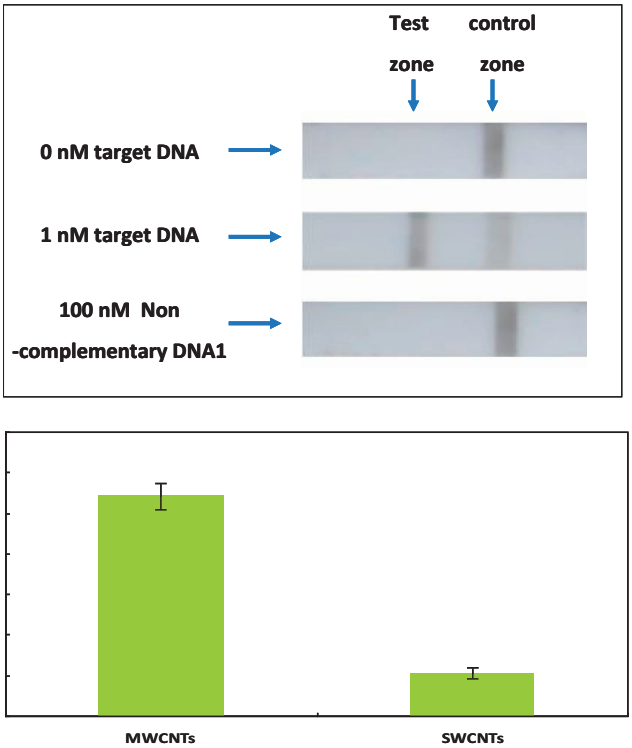

Fig. 3. (A) Typical images of MWCNT-based LFB in the absence and presence of 1 nM target DNA and 100 nM noncomplementary DNA; (B) signal-to-noise ratios of MWCNT- and SWCNT-based LFBs.

3.3. Optimization of the LFB parameters

To obtain the best performance of MWCNT-based LFB, analytical parameters, including running buffers, the concentration of the amine-modified detection DNA probe for preparing MWCNTs–DNA conjugates, the number of capture DNA probes on the test zone, and the volume of the MWCNT–DNA on the conjugate pad were optimized. First, we studied the effect of running buffers on the *S*/*N* ratio of LFB. Sample solutions were prepared by diluting the target DNA stocking solution with the buffers. Fig. 4A presents the histogram for the *S*/*N* ratios of LFB via the buffers. It can be seen the highest *S*/*N* ratio was obtained with 1/4 SSC+2% BSA, which was then used as running buffer in the following assays.

The amount of detection DNA probe on the MWCNT would affect the hybridization efficiency between the target DNA and detection DNA probe, and thus the performance of LFB. We optimized the concentration of the amine-modified detection DNA probe for preparing MWCNTs–DNA conjugates. As seen from Fig. 4B, the *S*/*N* ratio increased up to 0.1 OD mL^{-1}, then saturated till 0.3 OD mL^{-1}; a further increase in concentration caused a decrease in the *S*/*N* ratio. The *S*/*N* ratio loss at a high concentration may be attributed to stereo hindrance from the high density of detection DNA probe on the CNT surface.

The amount of capture DNA probes immobilized at the test zone also affects the LFB response. In the current study, the amount of capture DNA was optimized by dispensing different amount of streptavidin-biotinylated DNA complexes, which was done by changing the dispensing cycles on the test zone. Fig. 4C presents the effect of the capture DNA amount on the LFB's *S*/*N* ratio. The highest *S*/*N* ratio was obtained with 1 dispensing cycles, which was then used in the following assays. The decreased *S*/*N* with more dispensing cycles resulted from the increased background signal.

The intensity of test and control lines were greatly influenced by the amount of MWCNT–DNA conjugates dispensed on the conjugate pad. To obtain the best *S*/*N* ratio, the MWCNT–DNA on the conjugate pad was optimized by increasing the volume of the MWCNT–DNA conjugates loaded on the conjugate pad. Fig. 4D presents the histogram for the *S*/*N* ratio of LFB via the conjugate

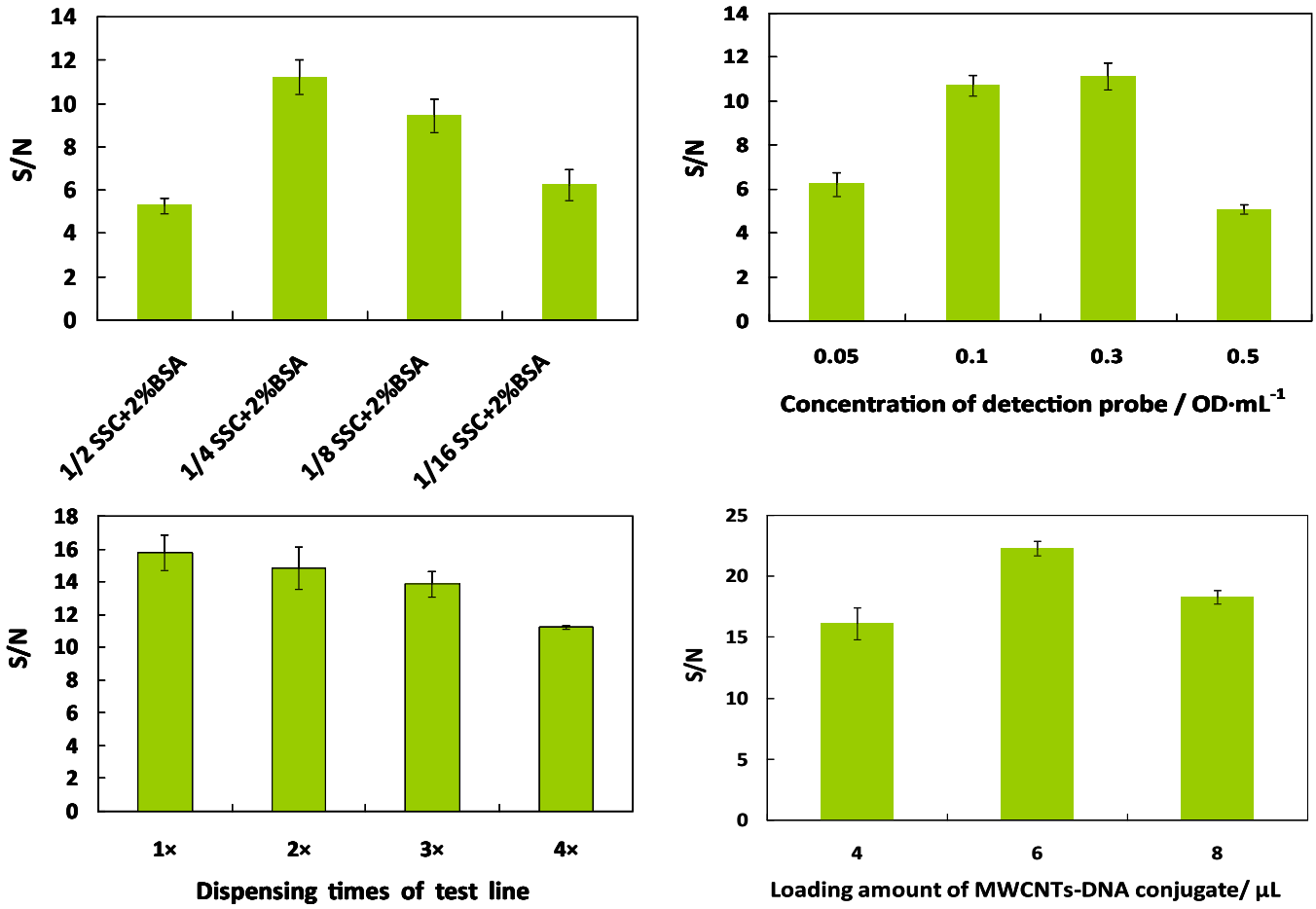

Fig. 4. (A) Effect of running buffers on the MWCNT-based LFB's *S*/*N* ratio; (B) effect of detection DNA probe concentration in MWCNT–DNA conjugation solution on the MWCNT-based LFB's *S*/*N* ratio; (C) effect of dispensing times of capture DNA probe on MWCNT-based LFB's *S*/*N* ratio; (D) effect of loading volume of MWCNT–DNA conjugate on the MWCNT-based LFB's *S*/*N* ratio. Target DNA concentration: 5 nM.

volume. The *S*/*N* ratio increased up to 6.0 μL; a further increase in volume caused a decrease in the *S*/*N* ratio. The *S*/*N* ratio loss at a larger volume may be attributed to a saturation of signal intensity and an increased nonspecific adsorption. Therefore, 6.0 μL of MWCNT–DNA conjugate was employed as the optimal volume throughout the entire study.

3.4. Analytical performance

Under optimal experimental conditions, we examined the performance of the MWCNT-based LFB in the presence of different concentrations of target DNA. Each sample was detected 3 times and the average value of three measurements was used to plot the

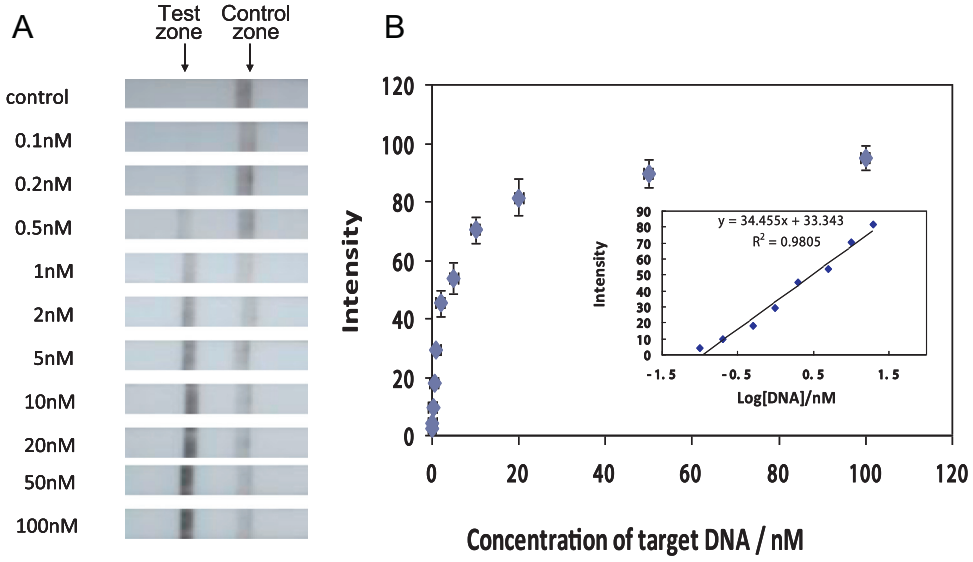

Fig. 5. Typical photo images (left) of the LFBs in the presence of different DNA concentrations and the resulting calibration curve (right).

calibration curve. Fig. 5A presents the typical photo images of MWCNT-based LFB after testing the different concentration of target DNA. There was no band observed in the control test, indicating negligible non-specific adsorption under the optimized experimental conditions. The intensity of the test band increased with the increase of target DNA concentration up to 20 nM, then saturated at the higher concentration. The test band was quite visible, even in the presence of 0.1 nM target DNA, which can be used as a threshold for the visual detection of DNA. Quantitative detection was also performed by recording the peak intensities of the bands on the test zone by using the Image J viewer software. The resulting calibration curve shows that the peak areas versus logarithm of the target DNA are linear over the 0.1–20 nM range (Fig. 5B), with a detection limit of 0.04 nM (40 pM), which is 12.5 times lower than that of GNP-based LFB (Mao et al., 2009).

3.5. Reproducibility, stability and specificity

In addition to high sensitivity, the MWCNT-based LFB also exhibited high reproducibility. The reproducibility of the LFSB was assessed by testing the LFBs in the absence and presence of 5.0 nM and 50 nM target DNA. Samples at the same concentration levels were tested 6 times, and one could see that similar responses were obtained at the same concentration levels (Fig. S1). The relative standard deviations (RSD) of the signal for control, 5 nM and 50 nM were 8.0%, 5.0%, and 3.6%, which indicates a good reproducibility of the measurements. The stability of the MWCNT-based LFB was tested by storing the LFBs at room temperature. It was found that their responses did not change significantly after one month′s storage at room temperature. The RSD of the LFB for 5 nM of target DNA was less than 8% compared with that obtained with the newly prepared LFBs, indicating the MWCNT-based LFB has a good stability. To confirm the specificity of the MWCNT-based LFB, sample solution containing non-complementary DNAs and the mixture of target DNA and noncomplementary DNA were tested. The results indicated that negligible responses were obtained in the presence of noncomplementary DNAs, and the high concentration of noncomplementary DNA did not affect the response of target DNA (Fig. S2). We also tested the response of one-base mismatched DNA on the LFB, unfortunately the MWCNT-based LFB couldn′t differentiate one-mismatched DNA and perfect-matched DNA (results not shown). Further work will aim to introduce the hairpin DNA probe as detection probe for one-base mismatched DNA detection.

4. Conclusions

We have developed a MWCNT-based LFB for sensitive and rapid detection of DNA. The sensitivity of the MWCNT-based LFB was enhanced 12.5 times compared to the previous GNP-based LFB. After systematic optimization, the MWCNT-based LFB was capable of detecting 40 pM DNA. Moreover, the use of MWCNT labels avoided the aggregation of conjugates, which was often met in the traditional gold nanoparticle-based LFB. The MWCNT-based LFB thus open a new door to prepare a new generation of LFB, and shows great promise for in-field and point-of-care diagnosis of genetic diseases and for the detection of infectious agents. The concept should be extended to visually detect protein biomarkers using the MWCNT-based LFB. Further work will aim to amplify the signal using enzyme-loaded MWCNT and detect miRNA in cell-lysate and biological fluids.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.028.

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