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Novel immunochromatographic assay on cotton thread based on carbon nanotubes reporter probe

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ABSTRACT

In this article, a novel immunochromatographic assay method on cotton thread based on carbon nanotubes (CNTs) as reporter probe was successfully prepared for visual and rapid detection of a lung cancer related biomarker, human ferritin antigen. A model system comprising ferritin as an analyte was used to demonstrate the protocol of the cotton thread immunoassay device. The device can detect at least 50 ng/mL human ferritin antigen, which improved the sensitivity approximately by 500 folds comparing with previous point-of-care test report based on carbon nanotubes as reporter probe. The CNTs reporter probe combined with cotton thread device based biosensor provided an alternative path for clinical diagnosis of other protein or nucleic acid biomarkers.

1. Introduction

Since carbon nanotubes (CNTs) were discovered by Iijima in the early 1990s, they have attracted considerable attention due to their unique physical, chemical, and electrical properties. CNTs have large surface area, and allow the adsorption of biomolecules for sensing [1–3]. In recent years, CNTs as reporter probe were applied in point-of-care test for detecting various diseases related biomarkers [4–8]. For example, Adeyabeba Abera [8] used lateral flow strip technology to detect human IgG utilizing carbon nanotubes as reporter probe, quantitative immunoassay response for the target human IgG was demonstrated in the range of 25–200 μg/mL, which indicated that the sensitivity of CNTs based point-of-care test for protein detection is not satisfied.

Recently, cotton thread has been reported as a support for transporting and mixing liquids for quantitative high-sensitivity immunoassays [9–11]. The construction and assay format of these cotton thread based immunoassay devices are similar with traditional immunochromatographic assay technology. Moreover, cotton thread has many special characteristics that make it a simple but powerful candidate for the fabrication of biomedical devices: (1) Flexible, lightweight, easy to manipulate and facilitate to transported or stored in any forms. (2) Low cost and broadly available with different characteristics (various hydrophilicities, colors, and sizes due to different pre-treatments). (3) The wicking properties of cotton threads can limit low volumes of sample solution from migration through it efficiently. (4) Easily to handle after use. Moreover, the assay format of the device is more like an immunochromatographic assay biosensor: sample solution and running buffer were dropped directly on the conjugate pad to rehydrate the conjugates. A series of our previous studies concentrated on cotton thread based on gold nanoparticles reporter probe have been reported recently [10,12,13], For example, Zhou [10] first introduced the immunochromatographic assay on cotton thread (ICAT) in a cartridge format that was suitable for multiplexing test. The ICAT was a sandwich assay performed on the cotton thread and we used three knotted threads as the substrate to detect C-reactive protein (CRP), osteopontin, and leptin proteins. In 2015, Du [13] introduced a dual label amplification protocol in the cotton thread based immunoassay device for low-cost, sensitive and rapid detection of a lung cancer related biomarker, squamous cell carcinoma antigen (SCCA). In the study, a dual labels amplification protocol has been employed for improving the detection sensitivity. All these studies used gold nanoparticles as reporter probe for immunochromatographic assays, to our best knowledge, there is no previous works about CNTs reporter probe based immunochromatographic assay on cotton thread device was reported.

In this work, we successfully developed a novel immunochromatographic assay on cotton thread utilizing carbon nanotubes as reporter probe for human ferritin detection. MWCNTs play a dual significant role in both as reporter probe and colored reagent. That means CNTs can be used as an efficient carrier for protein immobilization due to its large surface area. In the other hand, CNTs reporter probes solution

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show a dark black color, which can be used for visual test directly just as traditional gold nanoparticles. To perform an assay, in brief, a sandwich immune-complex would form on the test zone in the presence of human ferritin antigen, and a black band would be observed on the test zone, which indicated the accumulation of CNTs reporter probe on test zone, and can be used as a qualitative means. Quantitative detection can be realized by recording the color intensity of the test zone with a scanner and "Image J" software. Results indicated that the method reduced the detection limit by 500 folds comparing with previous point-of-care test report based carbon nanotubes as reporter probe, experiments also indicated that the sensitivity was improved undoubtedly comparing with traditional individual gold nanoparticles reporter probe.

2. Experimental section

2.1. Reagents and materials

Cotton threads (100% mercerized) were purchased from a cotton threads store (Xi'an). Human ferritin antigen and detection antibody (dAb), capture antibody (cAb) were supplied by Shanghai Linc-Bio Science Co., Ltd. Carcinoembryonic antigen (CEA) and squamous cell carcinoma antigen (SCCA) were supplied by Shanghai Linc-Bio Science Co., Ltd. Human serum and human immunoglobulin G (H-IgG) were purchased from Dingguo Biological Products (Beijing, China). Multi-walled carbon nanotubes (MWCNT) was purchased from Beijing DK nano technology Co., Ltd. (Beijing, China). Polyvinylpyrrolidone (PVP) was purchased from Dingguo Biological Products (Beijing, China). All other chemicals were of analytical reagent grade. All buffer solutions were prepared using ultrapure water.

2.2. Instrumentation

Basic pH meter was purchased from Sartoris (Beijing, China). Sterilization kettle was supplied by Shanghai Boxun Industry & Commerce Co., Ltd. (Shanghai, China). CanoScan 9000F was supplied by Canon Co., Ltd. (Thailand). Ultrasonic cleaner was applied by Ningbo xinzhi biotechnology Co., Ltd. (Ningbo, China). Drying oven was supplied by Yiheng technology Co., Ltd. (Shanghai, China). TG16-WS Desktop high-speed centrifuge was purchased from Hunan Xiangyi Laboratory Instrument Development Co., Ltd.

2.3. Preparation of CNTs–dAb reporter probe

Ten milligram of CNTs powder was refluxed for 24 h in 30 mL of 2.6 M HNO3. The solution was kept overnight, and the clear solution above the suspension was removed. The remaining suspension was centrifuged, and the precipitates was washed with deionized water till neutrality and dispersed in 20 mL deionized water. The carboxy-terminated CNTs solution was stored in 4 °C.

In order to start the conjugation process, 1 mL carboxylated of CNTs solution with 0.15 mg of PVP was sonicated for 4 h in an ultrasonic bath at 27 °C to exfoliate the CNTs and accelerate the dispersion. Then 8 μL 2.5 mg/mL monoclonal detection antibody against human ferritin was added into the solution (pH=8.2) to incubate for 12 h at 4 °C, and next added in 100 μL 10% BSA with stirring at room temperature for 1 h, finally was centrifugated at 12,000 rpm for 30 min to discard the supernatant. The precipitated CNTs-dAb was resuspended in 1 mL of PBST (0.75% Tween-20). The obtained CNTs-dAb reporter probe was stored at 4 °C for future use.

2.4. Construction of cotton thread immunochromatographic assay device based on CNTs reporter probe for determination of ferritin

The construction of cotton thread immunoassay device is simple. The cotton thread was pasted on two parallel placed double faced adhesive tapes, and a sample pad and an absorbent pad were applied at each end. The test zone in the middle of the cotton thread which was coated with 0.75 μL (applied as three aliquots of 0.25 μL, with 10 min drying at 37 °C after each step) of capture antibody against human ferritin at a concentration of 2.4 mg/mL, and then dried at 4 °C for 24 h. An absorbent pad (2×1 cm) was placed at one end of the cotton thread to serve as a capillary pump to draw the liquid. The sample pad was covered on the other end of the cotton thread. To perform an assay, a 75 μL portion of sample solution containing a desired concentration of ferritin antigen in running buffer (0.01 M PBS containing 0.75% Tween-20) was added into the sample pad and the solution would migrate toward the absorption pad. The test zone was evaluated visually within 25 min. For quantitative measurements, the optical intensity of the black band was read using the scanner combined with "Image J" software. For detecting ferritin in complex biological matrixes, such as human serum, CNTs reporter probe was centrifuged and the precipitated CNTs-dAb was dispersed in a mixture containing 37.5 μL of serum and 37.5 μL of running buffer (PBST). Other procedures are similar to above mentioned procedures.

3. Results and discussion

3.1. The cotton thread immunochromatographic assay device based on CNTs reporter probe for ferritin detection

The principle of CNTs as reporter probe for ferritin detection is based on the cotton thread immunoassay device as illustrated in Fig. 1. In this study, the cotton threads used in our experiments measure 600 μm in diameter, multi-walled carbon nanotubes (MWCNT) with the length of 10–30 μm and out diameter < 8 nm. As can be seen in Fig. 1, a cotton thread was attached on two parallel placed double faced adhesive tapes, and the test zone in the middle of the cotton thread was coated with capture antibody, an absorbent pad was applied at the downstream end to serve as a capillary pump to draw the liquid, a glass fiber was pasted on the other end of the cotton threads to serve as sample pad. To perform an assay, ferritin solution with desired concentration will react with detection antibody immobilized on the CNTs in centrifuge tube for 30 min at room temperature then was applied on the sample pad. The mixed solution containing the formed CNTs-dAb-Ag complexes would migrate along the cotton thread because of the capillary action, and would be captured by capture antibody pre-immobilized on the test zone of cotton thread to form a sandwich CNTs-dAb-Ag-cAb immune-complexes. The wicking properties of cotton threads limit low volumes of sample solution from migration through it efficiently, just like liquid migration in microfluidic channels caused by capillary action. Therefore, a black band on the test zone due to the accumulation of CNTs is visualized (Fig. 1(B)). The intensity of the resulting color band should vary proportionally to the concentration of ferritin antigen. The assay results were visible to the eye and were quantified using a commercial scanner. A scanner is an economic alternative to a densitometer that is commonly used to read lateral flow tests.

According to reference [5], the author also employed carbon nanotubes reporter probe on lateral flow strip for competitive immunochromatographic assay protein biomarker. The main component of the lateral flow strip was made of nitrocellulose membrane, and the width of the nitrocellulose membrane is about 0.3 cm, which is much wider than the cotton thread we used. The narrow size of the cotton thread means that the capture antibody located in the test zone has much higher concentration. In the meanwhile, CNTs reporter probe solution was restricted to fluid through the narrow cotton thread test zone, which result more CNTs reporter probes being captured in the test zone. Secondly, above mentioned study used a competitive immunoassay format to detect protein biomarker, while a sandwich immunoassay format was adopted in our method. That means the results obtained in our method were proportional to the concentration

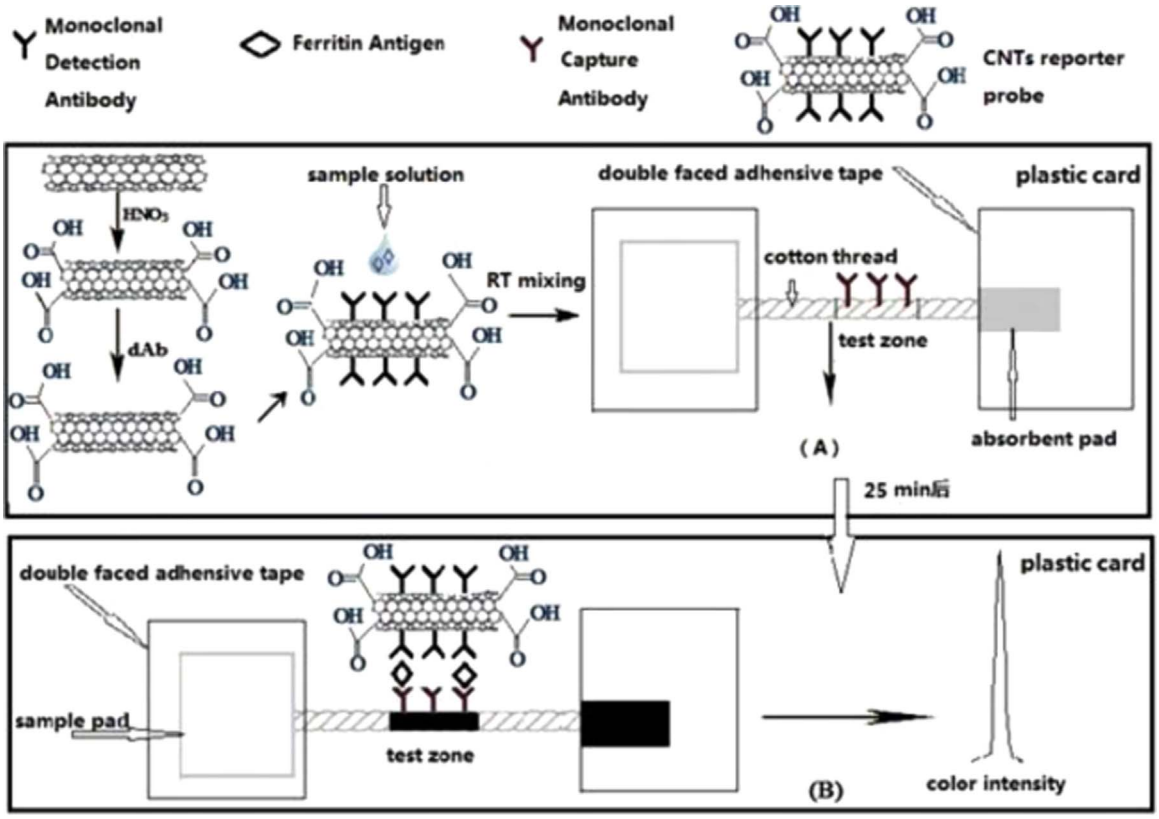

Fig. 1. The principle of the carbon nanotubes (CNTs) reporter probe based cotton thread immunochromatographic assay device for determination of human ferritin.

Table 1
Comparison of our work with previous report based on CNTs probe.

Contrast	Reference [5]	This work
Reporter probe	CNT	CNT
Analyte	Human IgG	ferritin
Assay format	competitive immunoassay format	sandwich immunoassay format
main component of the device	nitrocellulose membrane	cotton thread
modification of detection antibody protocol	typical EDC activation	physical adsorption
sensitivity	25 μg	50 ng

of protein biomarker directly. Thirdly, the modification of detection antibody protocol of our method is totally different with that of the previous study. In our work, carbon nanotubes surface carboxyl groups and carbon nanotubes defect level would be increased after acid oxidized pre-treatment [14]. As a result, detection antibody was easier and stronger to be modified on the surface of CNTs. Meanwhile, our experiments results indicated that the physical adsorption modification protocol is more efficient than the typical EDC activation protocol adopted by the previous study. All differences were listed as Table 1.

All these differences made the method developed here is much more sensitive, and the sensitivity was improved by 500 folds than the previous reported method (25 μg/mL). Moreover, the developed biosensor enables visual detection of human ferritin with naked eyes which avoid complicated electrochemical test procedures used in previous study.

Typical photo-images of cotton thread devices based on GNPs as reporter probe (A), CNTs as reporter probe (B) and corresponding peaks (C) in the presence of 0 and 5000 ng/mL of ferritin are shown in Fig. 2. As can be seen in Fig. 2(B), black bands will appear on the test zone of cotton threads in the presence of ferritin while no black bands can be observed in the absence of ferritin. Further more, red bands on the test zone of cotton threads based on gold nanoparticles as reporter probe hardly can be observed even in the presence of ferritin, while black bands on the test zone of cotton threads based on CNTs as reporter probe can be easily observed which can be explained at the advantages of CNTs that due to their internal characteristics. Identified peaks corresponding to Fig. 2(B) were recorded in Fig. 2(C). The former two peaks were obtained in the absence of human ferritin, while the last two peaks were recorded in the presence of 5000 ng/mL of human ferritin. The former two peaks were much lower than the last two peaks. The peak area was used as a quantitative parameter for ferritin test. The presence of the former two peaks is due to the difference between the white cotton thread and white paper, while these peaks are small, and constant. Summary, CNTs as reporter probe has a strong application potential in immunochromatographic assay method or cotton thread based immunoassay device to detect various diseases related protein biomarkers.

3.2. Optimization the detection conditions

3.2.1. Effect of volume of capture antibody on the test zone based on CNTs reporter probe

The response of the device is relevant to the volume of immobilizing capture antibody on the test zone. As shown in Fig. 3, the test zone was decorated with different amount (applied as three aliquots of 0.25 μL, with 10 min drying at 37 °C after each step) of capture antibody against human ferritin at a concentration of 2.4 mg/mL, and then dried at 37 °C for 1 h in drying oven. The volume of 2.4 mg/mL capture antibody on the test zone were 0.5, 0.75, 1 μL, respectively. The results displayed that the background (Black bars) and corresponding responses (Gray bars) of the device would enhance with increase of capture antibody repeat dropping times. The signal to noise ratio (S/N) was shown to be the highest for the total volume of capture antibody is 0.75 μL. The S/N would reduce if the repeat dropping times is further increased due to the background would elevate. Therefore, 0.75 μL was

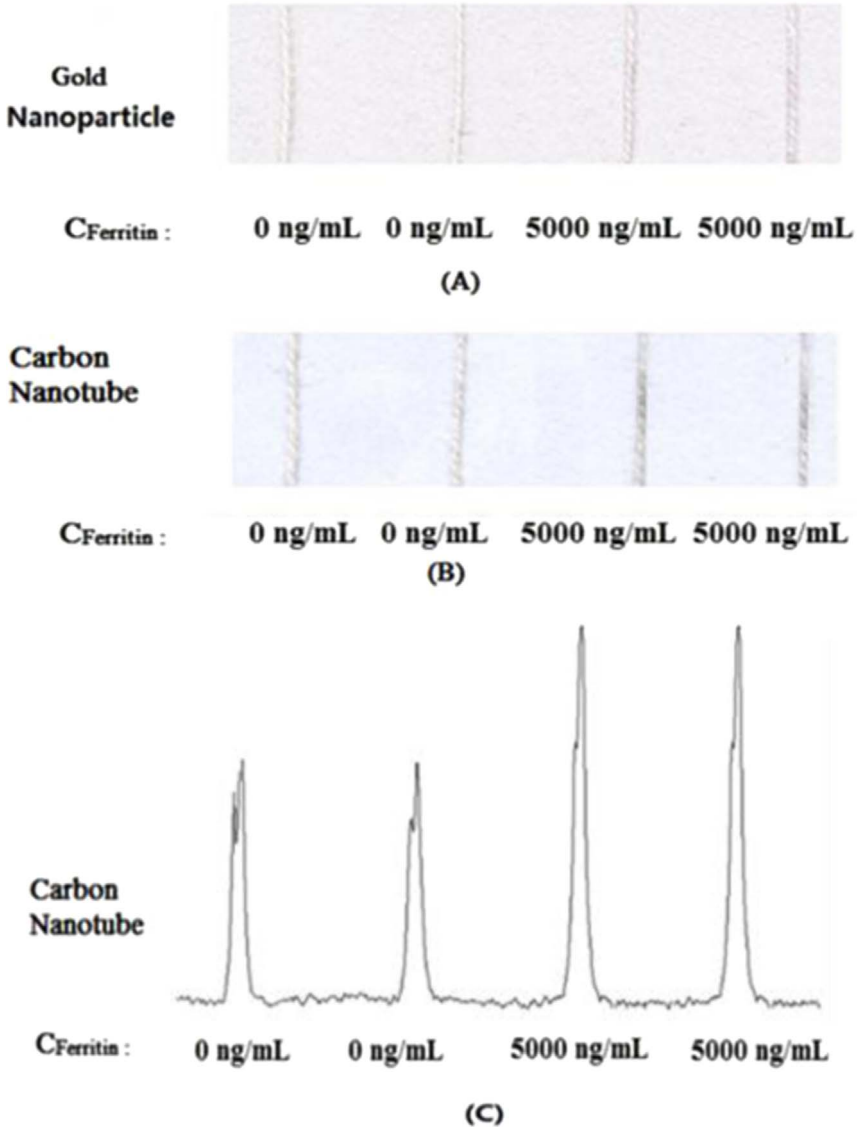

Fig. 2. Typical photo images of the cotton thread immunochromatographic assay devices based on gold nanoparticles as reporter probe (A), carbon nanotubes as reporter probe (B) and corresponding peaks (C) in the presence of 0 and 5000 ng/mL human ferritin.

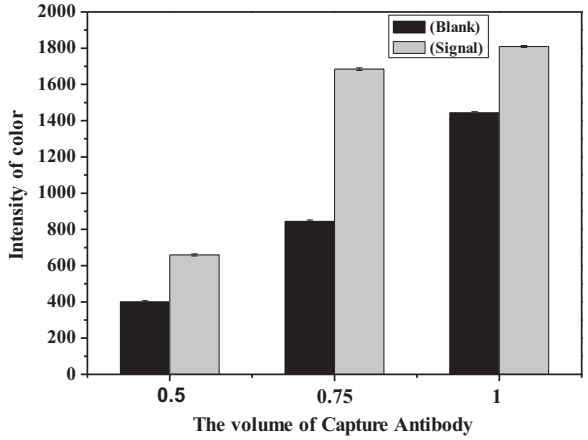

Fig. 3. Effect of volume of capture antibody on the test zone. The concentration of capture antibody is 2.4 mg/mL. The Black bars represents background in the buffer solution of PBST and Gray bars represents corresponding responses in the presence of 5000 ng/mL human ferritin antigen. (From left to right, the volume of capture antibody was: 0.5, 0.75 and 1 μL, respectively).

selected as the optimal amount of capture antibody in the following experiments.

3.2.2. *Effect of CNTs reporter probe amount*

The amount of CNTs reporter probe is also important to the sensitivity of the device. The amount CNTs reporter probe solution used will improve the reaction efficiency to generate more immune-complex and enhance the color intensity on the test zone. However, an excessive amount of CNTs reporter probe would result in both enhancement of response of the device and background that could ascribe to channel obstruction of the cotton thread. On the other hand, a small quantity of CNTs reporter probe will weaken the binding efficiency among CNTs reporter probe, human ferritin antigen and capture antibody, which will much weaken the intensity of the test zone. As shown in Fig. 4, with the elevating of CNTs reporter probe from 25 to 100 μL, maximal responses of the device was achieved at a volume of 75 μL. Thus, the CNTs reporter probe with a volume of 75 μL was used for the following experiment.

3.3. *Effect of other proteins*

Fig. 5 presents the response histograms of the cotton thread

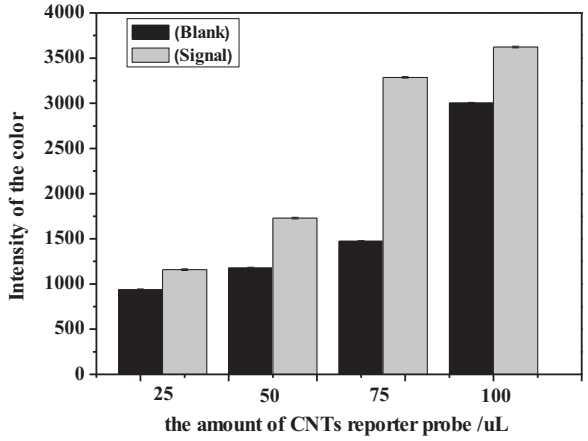

Fig. 4. Effect of CNTs reporter probe amount. The volume of capture antibody is 0.75 μL. The concentration of human ferritin antigen is 5000 ng/mL. From left to right, the volume of carbon nanotube reporter probe was 25, 50, 75 and 100 μL, respectively.

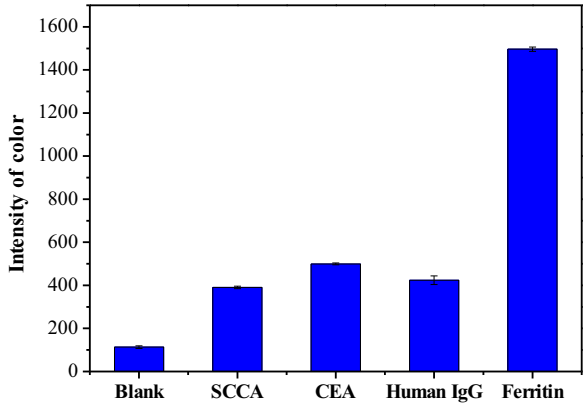

Fig. 5. Responses of the CNTs reporter probe based cotton thread immunochromatographic assay device with sample solution containing PBST (Blank), 5 μg/mL SCCA, CEA, human IgG and ferritin.

Table 2
The results of ferritin detection in serum.

Classification	Ferritin (ng/mL)
Healthy subjects	89.4 ± 24.5
Benign lung disease	140.3 ± 70.8
Patients with lung cancer	498.8 ± 87.3

immunochromatographic assay device based on CNTs reporter probe in the presence of running buffer (Blank), 5000 ng/mL of SCCA, CEA, human IgG and ferritin. A high response was observed when 5000 ng/mL ferritin was tested, whereas negligible signals were obtained in the absence of ferritin (Blank) and presence of 5000 ng/mL of SCCA, CEA or human IgG, which indicated the excellent selectivity of the CNTs reporter probe based cotton thread immunoassay device.

3.4. Analytical performances

To test the practicability of the device, experiments were performed by detection of ferritin in human serum (Fig. 6). Well-defined peaks were observed with a commercial scanner and "Image J" software, and the peak areas increased with the increase of ferritin concentration (Fig. 6(B), curves a-f). The resulting calibration plot (Fig. 6(A)) of the logarithm of peak areas versus ferritin concentration is linear over the range of 100~5000 ng/mL and is suitable for quantitative work. The detection limit of 50 ng/mL (based on S/N=3) was estimated within 25 min (data not shown). The specific response was coupled with high reproducibility. A series of measurements of 1 μg/mL human ferritin with well prepared CNTs based cotton thread immunochromatographic assay device yielded a reproducible signal with a relative standard deviation (RSD) of 9.6% (data not shown). The Table 2 presented the human ferritin level results for health, benign lung disease and lung cancer patients [15]. These comparison clearly demonstrated that the sensitivity of our developed method (LOD: 50 ng/mL) has reached the clinical requirement for lung cancer early diagnosis.

4. Conclusion

In conclusion, we introduced a novel immunosensor utilizing CNTs as reporter probe on the cotton thread immunochromatographic assay

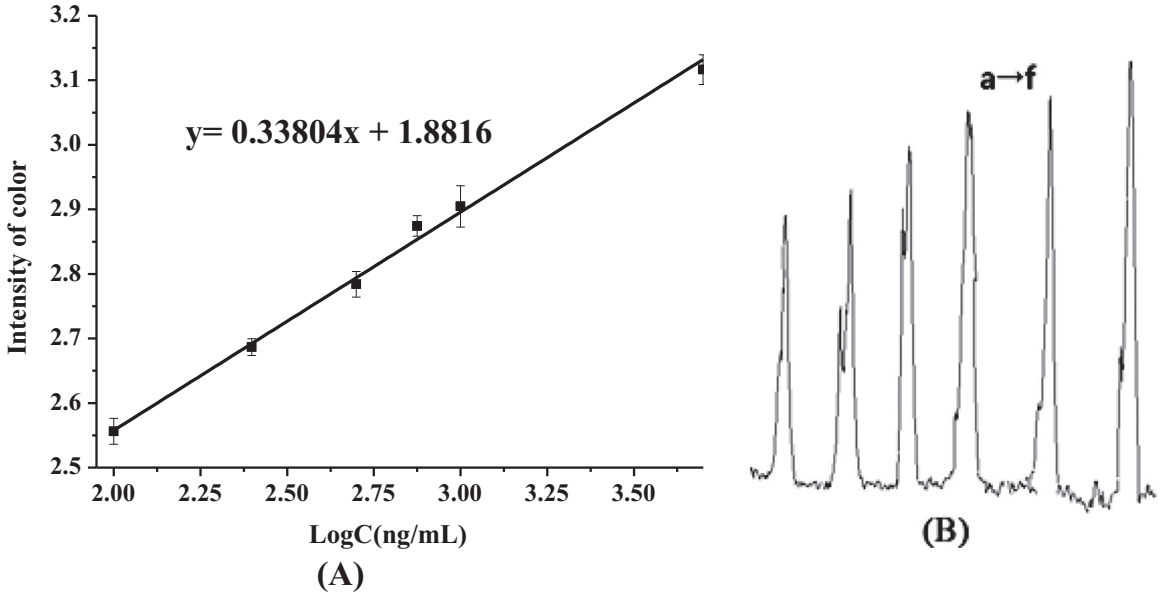

Fig. 6. The resulting calibration curve (Left) of the cotton thread immunochromatographic assay device by employing carbon nanotubes reporter probe in which the precipitated CNTs-dAb was dispersed by the mixture of human serum and PBST in the presence of different concentration of human ferritin and corresponding peaks (Right) under optimal conditions. From a to f: 100, 250, 500, 750, 1000 and 5000 ng/mL.

device for simple and rapid detection of human ferritin antigen. These special protocols greatly simplified assay procedures for ferritin test due to assay format is just like traditional lateral flow strip. Purification of modified treatment removed impurities, shorted length of carbon nanotubes, made its surface hydroxyl, carboxyl and carbonyl content significantly increased. Besides, with this biosensor, we can visually detect human ferritin with 50 ng/mL in sample solution within 25 min without instrumentation, which indicated more power for nearly 500 folds lower detect limit than that the similar report [8]. Therefore, the internal characteristics of CNTs made it an ideal candidate for protein test. Future works will focus on developing carbon nanotubes composites to improve detection sensitivity for proteins based on cotton thread device.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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