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An immunochromatographic assay for carcinoembryonic antigen on cotton thread using a composite of carbon nanotubes and gold nanoparticles as reporters

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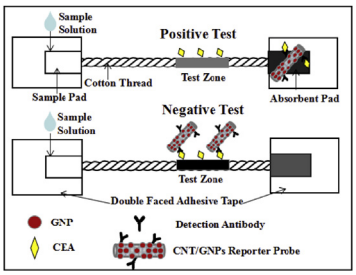
HIGHLIGHTS

- Novel carbon nanotube/gold nanoparticles nanocomposite probe based point-of-care diagnosis device.
- Visual carcinoembryonic antigen detection.
- The nanocomposite probe shows higher sensitivity comparing with carbon nanotubes or gold nanoparticles probe.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper describes a low-cost, sensitive, visual and rapid immunochromatographic assay method on cotton thread for carcinoembryonic antigen (CEA) detection by using novel carbon nanotube/gold nanoparticles (CNT/GNPs) nanocomposite reporter probe. CEA, a lung cancer protein biomarker, was used as analyte to demonstrate the principle of the immunochromatographic assay on cotton thread biosensor. In the presence of target CEA, the decreasing aggregation amount of CNT/GNPs nanocomposite reporter probes on the test zone induced directly readout by naked eye. Meanwhile, quantitative detection could be performed conveniently with a commercial available scanner. The performance with respect to sensitivity of the method was greatly improved by 2–3 magnitudes comparing with traditional gold nanoparticles (GNPs) or carbon nanotubes (CNTs) as reporter probe. Under optimal conditions, the biosensor was capable of detecting 2.32 ng/mL CEA (S/N $\geq$ 3) which is sensitive enough for clinical diagnosis. These results indicated the novel CNT/GNPs nanocomposite reporter probe based immunochromatographic assay on cotton thread is particularly suitable for point-of-care (POC) diagnostics in resource-limited regions.

1. Introduction

In the last two decades, nanomaterials, such as carbon based nanomaterials and noble metal nanoparticles, have captured the interest of researches worldwide in the field of point-of-care tests due to their unique properties [1]. Carbon nanotubes (CNTs) have

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been promising building block for biosensor design because of the small size with large surface area, high electrical conductivity, chemical stability and mechanical strength [2,3]. Point-of-care tests based on CNTs provide a simple and rapid approach for the detection of DNA, protein and glucose [4–9]. For example, Choi has reported the quantitative lateral flow immunosensor using carbon nanotubes as label for human immunoglobulin G detection with a minmal analyte concentration of 25 μg/mL [8]. All these reports indicated that CNTs are capable of applying in the field of point-of-care tests, but are still limited to achieve high sensitivity detection for protein biomarker test.

In recent time, natural cotton thread as an attractive and inexpensive material has been investigated as a suitable candidate for the fabrication of low-cost and low-volume microfluidic sensors to conduct biomedical and environmental assays [10]. For example, cotton thread has been reported by Whitesides and Shen as a matrix for making rapid biomedical assays and microfluidic diagnostics and rapid diagnostic tests [11,12]. Juncker has reported that cotton thread could be used as a support to transfer and mix analyte sample for immunochromatographic assays [13,14]. In our previous works, we used cotton thread immunochromatographic assay devices to achieve the quantitative detection of proteins and nucleic acid [15–17]. However, all these cotton thread immunoassay devices indicated that tranditional gold nanoparticles reporter probe without further amplifications possessed low sensitivity in point-of-care diagnosis and reliable applications.

Numerous biosensors based on CNT/GNPs nanocomposite in connection with different transducers (electrochemical, surface-enhanced Raman scattering (SERS), etc.) have been reported [18,19]. In this paper, CNT/GNPs nanocomposite reporter probe was used to combine with competitive immunochromatographic assay on cotton thread for rapid, sensitive and quantitative detection of CEA, which are successfully applied to overcome the above mentioned disadvantages of individual CNTs or GNPs reporter probe and achieve higher test sensitivity. The enhanced immunochromatographic biosensor consists of three parts: a sample pad, a cotton thread, and an absorbent pad. A certain concentration of CEA was immobilized onto the cotton thread to form the test zone. Detection antibody (dAb) was coated on the surface of GNPs of CNT/GNPs to form a nanocomposite reporter probe. In the presence of target CEA, the decreasing amount of CNT/GNPs nanocomposite accumulates on the test zone by the competition of CEA in sample solution and pre-immobilized on the test zone, and a weaker color band can be observed on the test zone. The remaining CNT/GNPs nanocomposite continue to migrate toward to absorbent pad. While CNT/GNPs nnaocomposite aggregates greatly and a distinct color band can be observed on test zone in the absence of specific analyte. Quantitative detection was performed by recording the color intensity changes of the test zone with a scanner and "ImageJ" software.

2. Experimental section

2.1. Reagents and materials

Cotton thread (100% mercerized) was purchased from a thread store (Xi'an). CEA and dAb were obtained from Sangon Biotech (Shanghai, China). CNTs (purity ≥ 95%, diameter 30–50 nm, and length 10–20 μm) was applied by Kededaojin Co. Ltd. (Beijing, China). Poly (diallyldimethylammonium chloride) (PDDA, 20%, w/w in water, MW: 200 000–350 000) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Albumin bovine serum (BSA) and human immunoglobulin G (H-IgG) was purchased from Dingguo Biological Products (Beijing, China). Squamous cell carcinoma antigen (SCCA) and Ferritin antigen were supplied by Shanghai Linc-Bio Science Co. Ltd. All other chemicals were of analytical reagent grade. Phosphate buffered saline (PBS, 0.01 M, pH 7.4) was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. PBS containing 1% BSA and 2.5% Tween 20 (PBSBT) was used as washing buffer, dispersion buffer and running buffer. Ultrapure water produced by a Milli-Q system applied by Millipore China Co. Ltd. (Shanghai, China) was used to prepare all buffer solutions.

2.2. Instrumentation

Sterilization kettle was purchased from Shanghai Boxun Industry & Commerce Co. Ltd. (Shanghai, China). TG16-WS high speed tabletop centrifuge was supplied by Xiangyi laboratory instrument development Co. Ltd. (Hunan, China). Drying oven was purchased from Yiheng technology Co. Ltd. (Shanghai, China). Ultrasonic clearing machine was supplied by Ningbo scientz biotechnology Co. Ltd. (Zhejiang, China). CanoScan 9000F was obtained from Canon Co. Ltd. (Thailand).

2.3. Synthesis of GNPs

GNPs with the diameter of 15 ± 3 nm was prepared according to the previous protocol [15]. Briefly, 2.5 mL 1% trisodium citrate solution was rapidly added into 50 mL stirred boiling solution of 0.01% $HAuCl_4$. After turning deep red, the resulting solution was boiled for another 15 min to ensure a stable color and then cooled to room temperature. The concentration of the GNPs is 0.06 mg/mL. The obtained GNPs solutions were stored at 4 °C for future use.

2.4. Synthesis of CNT/GNPs nanocomposite

The acid-treated CNTs were further functionalized with PDDA according to the documented method [18]. Then, the obtained PDDA functionalized CNT (0.5 mg) was dispersed in 6.0 mL of as-prepared GNPs and stirred for 30 min. After centrifugation, the obtained light purple CNT/GNPs nanocomposites were washed three times with ultrapure water and redispersed in 1.8 mL of 50 mM Tris-HCl (pH 9.0) solution. The obtained CNT/GNPs nanocomposites solution was stored at 4 °C for future use. The nanocomposites would become precipitate after keeping stable for approximately 1 h. However, the precipitates could be re-dispersed in solution after ultrasound treatment before use.

2.5. Preparation of CNT/GNPs nanocomposite reporter probe

The preparation of CNT/GNPs nanocomposite reporter probe is pretty simple. Firstly, 12 μL of 0.5 mg/mL dAb was added to 300 μL of CNT/GNPs solution and incubated at room temperature. After 5 min, 30 μL 10% BSA was added to the mixture, followed by gently mixing at 4 °C for 3 h and centrifuging at 5000 rpm for 10 min. After centrifugation, the CNT/GNPs nanocomposite reporter probe was obtained, which was thrice washed with PBSBT and redispersed in 300 μL PBSBT mentioned above as the assay solution. The obtained bioconjugate solution was stored at 4 °C for future use. Prior to use, this solution was immediately diluted 3.75-fold with PBSBT.

2.6. Construction of cotton thread device for CEA detection

The immunochromatographic assay device consisted of following components: a sample pad, a cotton thread and an absorbent pad. All of the components were mounted on a common backing layer using double faced adhesive tapes. Cotton threads were boiled by 2 M NaCl for 30 min, and then soaked in 0.01% H_2O_2 and 0.01 M HCl for 5 min, respectively, followed by washing with

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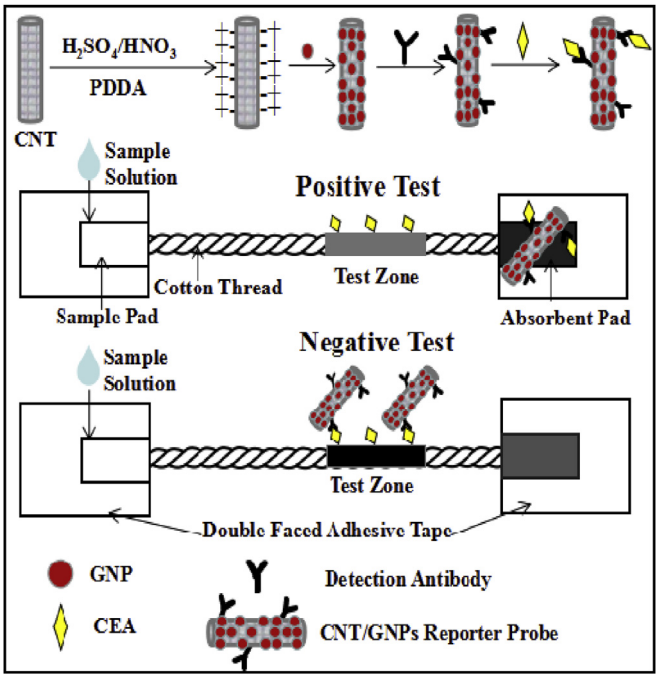
large amount of ultrapure water. Finally, cotton threads were dried at 37 °C and stored for further use. CEA of 0.75 μL (applied as three aliquots of 0.25 μL, with 10 min drying at 37 °C after each step) was dispensed onto the cotton thread to form test zone. The cotton thread then dried at 37 °C for 1 h in drying oven and stored at 4 °C until use. Double faced adhesive tapes were sticked to a clean plastic pad in parallel ways with a space wide about 2.5 cm. The filter paper strip as absorbent pad was attached at the downstream end of thread, the glass fiber as sample pad was covered on the other end of the thread. CEA with desired concentration incubated with the dAb labeled on the surface of CNT/GNPs nanocomposite for 30 min at room temperature to form sample solution. To perform an assay, the sample solution was loaded onto the sample pad. Then, running buffer (PBSBT) was following applied to the sample pad and the solution migrated along with cotton thread and toward the absorbent pad. The test zone was evaluated within 20 min by naked eye. According to the previous method [14], the quantitative measurements can be performed by recording the optical intensity of the color bands on the test zone using the scanner combined with "ImageJ" software.

3. Results and discussion

3.1. The principle of novel immunochromatographic assay on cotton thread

The principle of the novel CNT/GNPs nanocomposite reporter probe based immunochromatographic assay on cotton thread biosensor for CEA detection is based on the competition of a binding event between CEA in sample solution (unknown concentration) and known CEA (immobilized on the test zone of cotton thread) to the dAb on the surface of CNT/GNPs nanocomposite, and the protocol is illustrated in Scheme 1. CEA was immobilized on the cotton thread to form test zone. When sample solution containing CNT/GNPs nanocomposite reporter probe and target CEA loaded on the sample pad, the solution began to flow along the long axis of the cotton thread due to the capillary effect. The CNT/GNPs nanocomposite accumulate rarely on test zone because of the competition between CEA in sample solution and pre-immobilized on test zone. In this case, the color band on test zone weakened significantly due to the decreasing accumulation amount of CNT/GNPs. The color intensity of the color band was inversely proportional to the concentrations of the analyte concentration. In the absence of target CEA, there was a significantly deep color band on test zone, which indicated that the biosensor functioned correctly. As can be seen in Scheme 1, the above cotton thread device showed a positive test which the sample solution dropped on the sample pad containing analyte CEA and CNT/GNPs nanocomposite reporter probe, while the below one showed a negative test which the sample solution has CNT/GNPs nanocomposite reporter probe alone.



Scheme 1. Principle of novel CNT/GNPs nanocomposite reporter probe based immunochromatographic assay on cotton thread device.

Quantitative detection was performed by recording the color intensity changes of the test zone with a scanner and "ImageJ" software. Typical photo images (A) and corresponding responses of the cotton thread based device (B) in the presence of 0 and 5000 ng/mL of CEA are shown in Fig. 1. We used a white paper, whose color is similar to new clear cotton thread, as the base plate to photograph. For the three cotton threads involved in assays without CEA, deep color bands could be seen in the test zones (Fig. 1A), as well as higher corresponding color intensity peaks can be observed (Fig. 1B). The difference between the deep color bands on the test zones and white paper formed the higher peaks. For the three cotton threads involved in assays with CEA, weak color bands in the test zones (Fig. 1A) and lower corresponding color intensity peaks can be observed (Fig. 1B). Because of the exist of CEA in the sample solution, tiny amounts of CNT/GNPs nanocomposite reporter probes bound specifically with CEA on the test zone of cotton thread. Thus, the decreasing aggregation amount of CNT/GNPs nanocomposite reporter probes on the test zone of cotton thread lead to the presence of light color bands and lower corresponding color intensity peaks.

The TEM image and UV-vis absorption spectra of GNPs, CNTs and CNT/GNPs were presented in Fig. 2. The synthetic GNPs have uniform size about 15 nm (Fig. 2A). The oxidized CNT showed a homogeneous surface and good dispersion (Fig. 2B). GNPs could compactly attach on the surface of the CNTs by the PDDA bridge to form CNT/GNPs nanocomposite (Fig. 2C). Free GNPs were seldom to be observed. Fig. 2D presents the UV-vis absorption spectra of the CNT/GNPs suspension, CNTs solution, and GNPs suspension. An absorption peak at around 520 nm was observed for GNPs solution (Fig. 2D, a) and CNTs solution exhibited an absorption peak at 257 nm (Fig. 2D, b). However, CNT/GNPs solution showed two absorption peaks at around 534 nm and 268 nm, respectively, which indicated that CNTs and GNPs successfully formed nanocomposites

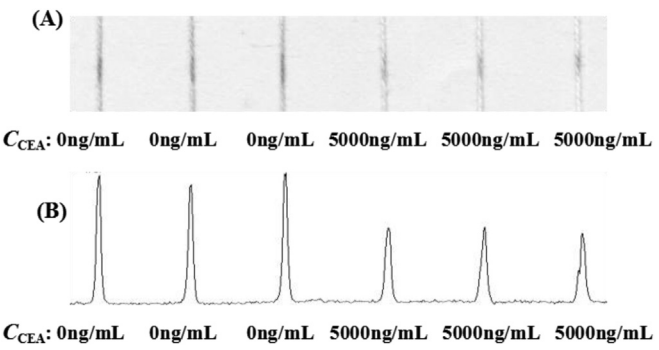


Fig. 1. Typical photo images (A) and corresponding responses (B) of novel CNT/GNPs nanocomposite reporter probe based immunochromatographic assay on cotton thread device in the presence of 0 and 5000 ng/mL CEA.

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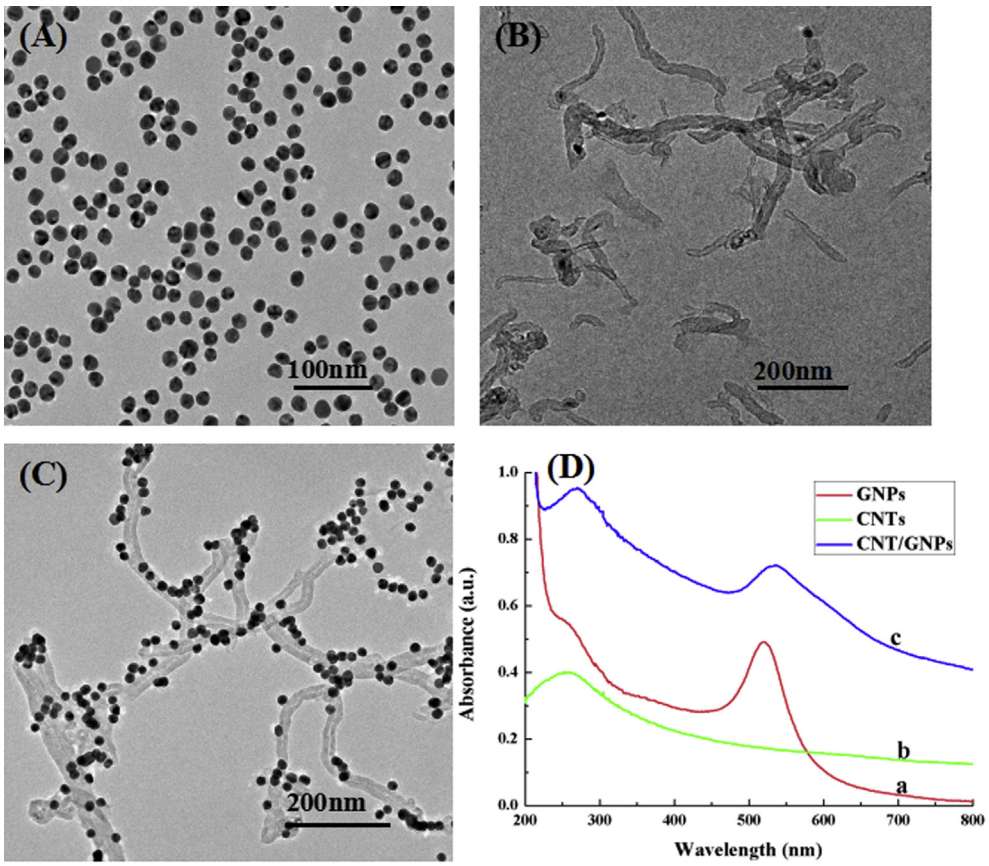

Fig. 2. TEM images of GNPs (A), CNTs (B) and CNT/GNPs (C) and UV-vis absorption spectra (D) of GNPs (a), CNTs (b) and CNT/GNPs (c).

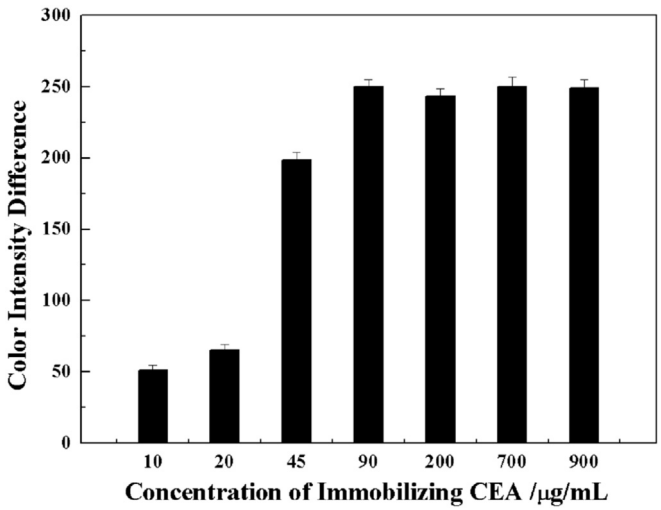

Fig. 3. Effect of immobilizing concentration of CEA. CEA concentration: 100 ng/mL.

(Fig. 2D, c). The red shift of the absorption peak of the nanocomposites may be due to both the size difference and the interaction between GNPs and CNTs.

3.2. Effect of immobilizing concentration of CEA

The concentration of CEA on test zone of cotton thread affects the immunochromatographic assay device response. As showed in Fig. 3, the difference between background and corresponding responses of the device would increase with the increasing of immobilized CEA concentration when the concentration was less than 90 μg/mL. However, when the immobilized CEA concentration was more than 90 μg/mL, the difference between background and corresponding responses would keep stable. The gradually increased color intensity difference is ascribed to the increased background value. Therefore, 90 μg/mL CEA was selected as the optimal immobilizing concentration in the following experiments.

3.3. Effect of amount of CNT/GNPs nanocomposite

The intensity of the color band on the test zone is closely related to the amount of CNT/GNPs nanocomposite. As can be seen in Fig. 4, the difference between background and corresponding responses increased first and then decreased with the increasing dosage of CNT/GNPs nanocomposite, and the difference was highest when the volume of CNT/GNPs nanocomposite was about 8 μL. Tiny amount led to lower background value, while larger amount resulted in increased signal, consequently decreasing the background value. Therefore, the volume of 8 μL CNT/GNPs nanocomposite was choosed in the experiment.

3.4. Effect of other proteins

To confirm the specificity of this assay for CEA detection, SCCA, human ferritin and human IgG were used as negative controls. As showed in Fig. 5, a high response difference was observed in the presence of 1000 ng/mL CEA, whereas negligible signal difference

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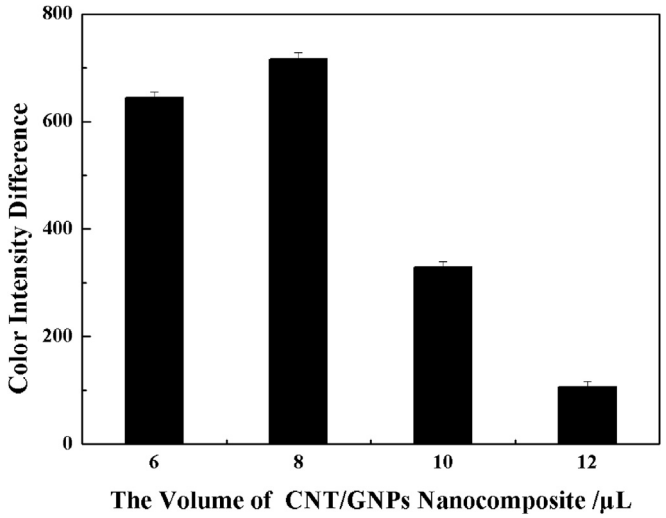

Fig. 4. Effect of amount of CNT/GNPs nanocomposite (6 μL, 8 μL, 10 μL, 12 μL), respectively. CEA concentration: 100 ng/mL.

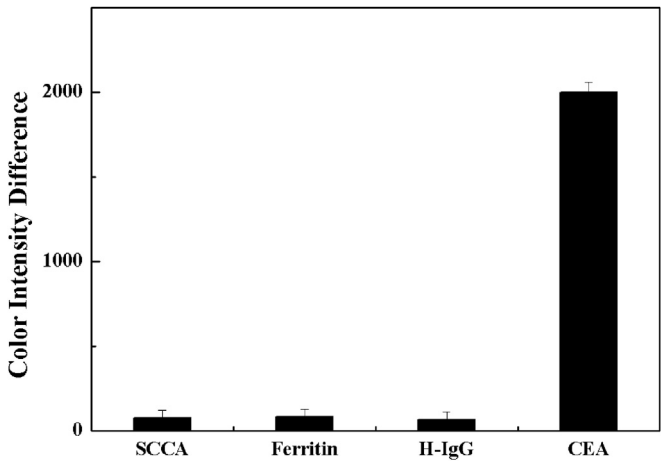

Fig. 5. Response of the novel CNT/GNPs nanocomposite reporter probe based immunochromatographic assay on cotton thread device in the presence of PBSBT (Blank), 1000 ng/mL SCCA, 1000 ng/mL human ferritin, 1000 ng/mL human IgG, and 1000 ng/mL CEA.

were obtained when 1000 ng/mL of SCCA, human ferritin, human IgG or PBSBT buffer solution loaded onto sample pad. The experimental results indicated that this method can successfully detect CEA without interference from other proteins.

3.5. Analytical performance

To evaluate the practicability of the device for detecting protein biomarkers, experiments were performed to detect CEA in human serum (Fig. 6). The sample solutions were prepared by spiking CEA into the mixture of human serum and PBSBT (1:5). As shown in the inset of Fig. 6, the peak areas decreased with the increase of CEA concentration in sample solution. The resulting calibration plot of the logarithm of peak areas difference between background and signal to CEA concentration is proportional in the range from 5 to 500 ng/mL (Fig. 6), the detection limit was estimated to be 2.32 ng/mL from 3 times the standard deviation corresponding to the blank sample detection (S/N = 3). Moreover, when detecting CEA in

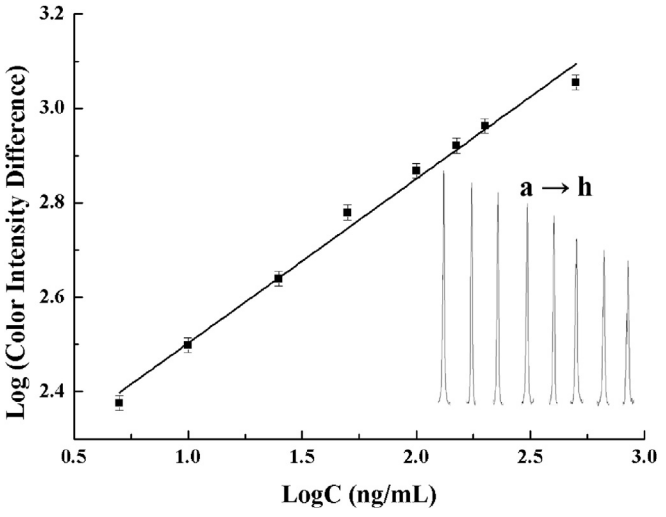

Fig. 6. The resulting calibration curve and corresponding peaks (inset) of the novel CNT/GNPs nanocomposite reporter probe based immunochromatographic assay on cotton thread device in various concentration of CEA in human serum sample solutions. From a to h: 5, 10, 25, 50, 100, 150, 200 and 500 ng/mL.

PBSBT, the response of the optimized device was highly linear for the range of 10–500 ng/mL with a detection limit of 2.36 ng/mL. The relative standard deviations (RSD) were estimated to be 4.82%, and 5.21% (data not shown) for 8 times detection of 100 ng/mL CEA in human serum and PBSBT, respectively, indicating an excellent reproducibility. These results proved that this assay can be successfully applied for detection of CEA and holds great potentials as an universal POC test approach for disease screening and cancer diagnosis.

3.6. Comparison of immunochromatographic assay on cotton thread performance with other methods

We compared the detection limit, assay time and linear range of immunochromatographic assay on cotton thread with other methods for protein detection (Table 1) [8,14,20–23]. As can be seen in Table 1, the current method can greatly improve sensitivity for protein detection by 4 magnitudes or 4-fold compared with conventional CNT-based lateral flow immunosensor and traditional GNP-based immunochromatographic assay on cotton thread. The comparison results indicates that individual CNTs or GNPs without further amplifications possessed relative lower sensitivity for POC tests, while CNT/GNPs nanocomposite has great potential to enhance performance for protein biomarkers tests because of the integration of the advantages of CNTs and GNPs. Comparing with piezoelectric immunosensor, Paper-based chemiluminescence ELISA and fluorescence method for CEA detection, detection limit of the proposed method increased greatly or equated. And colorimetric method has a high sensitivity when compared with current method. However, these two methods have great differences in detection instruments. The UV-VIS Spectrophotometry would be used in colorimetric method, which is expensive and complicated, while the current method only used a commercial available scanner to achieve quantification analysis. CEA cut-off value for lung cancer diagnosis is 10 ng/mL, thus the detection limit of 2.32 ng/mL of the current well-designed method has reached the requirement for clinical diagnosis, which indicated that the immunochromatographic assay on cotton thread has a very promising prospect in the

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Table 1
Summary of the analytic performances for protein detection with different methods.

Method	target protein	detection limit and assay time	linear range	ref
CNT-based lateral flow immunosensor	human IgG	25 μg/mL; 15 min	25-250 μg/mL	[8]
GNP-based immunochromatographic assay	CRP	9.82 ng/mL; 20 min	0-100 ng/mL	[14]
piezoelectric immunosensor	CEA	66.7 ng/mL; 40 min	66.7–466.7 ng/mL	[20]
Paper-based chemiluminescence ELISA	CEA	5 ng/mL; unknown	0.1–70 ng/mL	[21]
Fluorescence method	CEA	1 ng/mL; unknown	2.5–100 ng/mL	[22]
Colorimetric method	CEA	0.048 ng/mL; unknown	0.05–50 ng/mL	[23]
CNT/GNPs-based immunochromatographic assay	CEA	2.32 ng/mL; 20 min	5-500 ng/mL	current method

high sensitive POC detection of protein biomarkers.

4. Conclusions

In conclusion, a novel CNT/GNPs nanocomposite reporter probe has been prepared via layer-by-layer assembly for design of an immunochromatographic assay cotton thread device for CEA detection. CNT/GNPs nanocomposite reporter probe displayed super advantages in terms of sensitivity comparing with previous reported individual CNTs or GNPs reporter probe. This proposed method showed excellent performance for CEA detection with low detection limit. Furthermore, the sample solution required for device is low-volume due to the small size of cotton thread, sample pad and absorbent pad employed. The convenient operation and superior sensitivity of the analysis method provided a promising potential for other protein biomarkers tests in clinical diagnosis. Further works will focus on multiplexing detection of cancer biomarkers.

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