

Lateral flow assay for carbohydrate antigen 19–9 in whole blood by using magnetized carbon nanotubes

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Abstract The authors describe a rapid, low-cost and sensitive approach for the determination of carbohydrate antigen 19–9 (CA 19–9) in whole blood by using magnetized carbon nanotubes (MCNTs) and a lateral flow strip biosensor (LFSB). MCNTs were synthesized by depositing magnetite (Fe_3O_4) nanoparticles on multiwalled carbon nanotubes (CNTs) via co-precipitation of ferric and ferrous ions within a dispersion of shortened multiwalled CNTs. Antibody against CA 19–9 (Ab_1) was covalently immobilized on the MCNTs and were used to capture CA 19–9 in blood. After magnetic separation, the MCNT- Ab_1 -CA 19–9 complexes are applied to the LFSB, in which a capture antibody (Ab_2) and a secondary antibody (Ab_3) are immobilized on the test zone and control zone of the LFSB, respectively. The captured MCNTs on the test zone and control zone are producing characteristic brown bands, and this enables CA 19–9 to be visually detected. Quantitation is accomplished by reading the intensities of the bands with a portable strip reader. Under optimized conditions, the assay has a detection limit as low as $30 \text{ U} \cdot \text{mL}^{-1}$ of CA 19–9 in blood. This is below the cutoff value ($37 \text{ U} \cdot \text{mL}^{-1}$) of CA 19–9. The assay duration for blood samples is 35 min. In our perception,

the assay represents a rapid and low-cost tool for rapid determination of CA 19–9 in blood that holds promise for clinical applications, particularly in limited resource settings.

Keywords CA 19–9 · Visual detection · Magnetized carbon nanotube · Lateral flow assay · Cancer biomarker

Introduction

Carbohydrate antigen 19–9 (CA 19–9) is a cancer-related glycoprotein antigen with the molecular weight of 210 kD, which was first isolated in 1979 by Koprowski and coworkers from colorectal carcinoma and later from pancreatic carcinoma [1]. CA 19–9 is a well-established biomarker for pancreatic carcinoma, and has also been reported to be positive in other types of gastrointestinal cancers including gastric cancer, cholangiocarcinoma and colorectal cancer [2]. The level of CA 19–9 is also of great value for envisaging the prognosis of the disease, estimating the severity of the disease and predicting recurrence. Elevated levels ($>37 \text{ U} \cdot \text{mL}^{-1}$) of CA 19–9 have been associated with gastrointestinal carcinomas, particularly in pancreatic cancer, and CA 19–9 is considered one of the most promising biomarkers for the management of pancreatic cancer [3]. CA 19–9 is the only biomarker that has been cleared by the U.S. Food and Drug Administration (FDA) for prognosis and following the course of pancreatic cancer [4, 5]. Hence, sensitive and specific determination of low-abundant CA 19–9 in biological fluids would be advantageous in clinical diagnosis.

A variety of strategies and techniques have been developed to detect CA 19–9, including radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), western blot, agarose and polyacrylamide gel electrophoresis [6]. The gold standard for detecting CA 19–9 is ELISA [7]. Several ELISA kits

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(Invitrogen; Panomics; Diagnostic Automation Inc. and Fitzgerald) are commercially available for the determination of CA 19–9. The sensitivities of ELISAs have been further enhanced by using electrokinetic concentration chip [8] and microfluidic device [9]. Although ELISA kits and improved ELISAs have some advantages (ease of use, flexibility, and relatively low cost), they are time-consuming (about 3 h to get results) and require well-trained personnel. Immunosensor technology, as an alternative and simpler immunoassay method, has been used for fast and sensitive CA 19–9 analysis in connection with different transducers [10]. Various immunosensors have been reported to detect serum CA 19–9 [11–15]. Although the reported immunosensors have shown some promise to substitute for the traditional enzyme immunoassay of CA 19–9, most of them are being utilized at the laboratory research level and have not been applied in the field or with point-of-care applications due to their relative long assay time and their multiple washing and separation steps. To keep pace with expectations in future point-of-care testing and meet the urgent demand of sensitive and selective CA 19–9 detection in limited resource settings without costly equipment and well-trained personals, development of a rapid, low-cost and high accuracy detection method for disease diagnostics is highly desired.

Lateral flow immunoassay (LFI), also known as immunochromatographic assay or lateral flow strip biosensor (LFSB), is a solid-phase immunoassay incorporating the technology of thin layer chromatography and immune recognition reaction [16, 17]. Nowadays, nanoparticles-based lateral flow strip biosensor has attracted significant attention in biological analysis and clinical diagnosis [18–20]. It is one kind of rapid, cost-effectiveness, and portable detection technique which has been used as commercial products for point-of-care or in-field screening of infectious diseases, drugs of abuse, and pregnancy [21, 22]. Our group and others have successfully devised quantitative LFSBs for the detection of proteins and nucleic acids with the help of a portable strip reader [23–26]. Detecting CA 19–9 using LFSBs in blood without sample-pretreatment is still a great challenge because of problems such as biofouling and nonspecific binding, and resulting need to use sample purification greatly reduces the clinical applications [27]. Traditional GNP-based LFIs are not able to detect proteins with low concentrations in whole blood due to its low sensitivities and color interference. Fluorescent LFIs have been applied to detect proteins in whole blood because of their high sensitivities. Gerd et al. reported a lateral flow immunoassay using europium (III) chelate microparticles and time-resolved fluorescence for eosinophils and neutrophils in whole blood [28]. Own to unique magnetic separation properties, magnetic microparticles and nanoparticles have been used as immunochromatographic labels for the detection of biomarkers in blood [29]. However, fluorescent and magnetic LFIs still require expensive

or complex readers. Therefore, there is still a great challenge to develop inexpensive, rapid and easy-to-use technologies for CA 19–9 detection in whole blood.

In this study, the authors synthesized a magnetized carbon nanotube (MCNT) by coating Fe_3O_4 nanoparticles on the shortened multiwalled CNT surface via co-precipitation of ferric and ferrous ions within a dispersion of shortened multiwalled CNTs [30]. The anti-CA 19–9-modified MCNTs were used to capture CA 19–9 in whole blood. After a magnetic separation, the formed CA 19–9-anti-CA 19–9-MCNT complexes were applied to the LFSB, in which a capture antibody is immobilized on the test zone of the LFSB. The captured MCNTs on the test zone and control zone are producing characteristic brown bands, and this enables CA 19–9 to be visually detected. Quantitation was accomplished by reading the intensities of the bands with a portable strip reader.

Experimental

Apparatus

Nucleic Acid Extraction MCB 1200 (Sigris Research, Inc., Brea, California, <http://www.sigris.com/>) was used to separate magnetized carbon nanotubes from solutions. A Hitachi SU8010 field scanning-electron microscope (SEM; Tokyo, Japan, <http://www.hitachi-hightech.com/>) was used for images taking of the CNTs and MCNTs. Fourier transform infrared (FT-IR) spectroscopy measurements were measured using a Nicolet iS10 FT-IR Spectrometer (Thermo Scientific, Rockford, IL, <https://www.thermofisher.com/>) with attenuated total reflection (ATR) attachment. Raman spectrums were measured using an inVia-Reflex confocal Raman microscope (Renishaw, England, <http://www.renishaw.com>). The Biojet BJQ 3000 dispenser, Clamshell Laminator, and the Guillotine cutting module CM 4000 purchased from Biodot LTD (Irvine, CA, <https://www.biodot.com/>) were used to prepare lateral flow strip biosensors. A portable strip reader DT1030 (Shanghai Goldbio Tech. Co.; Shanghai, China, <http://www.kinbio.com/>) was used for signal recording. Nikon COOLPIX S4200 camera (Nikon, Japan, www.nikon.com/) was used to take the photo images of lateral flow strip biosensors.

Reagents

Multiwalled carbon nanotubes (MWCNTs, SN2302, purity > 95%) were purchased from Nanomaterial Store (Fremont, CA, <https://www.nanomaterialstore.com/>), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (purity > 99%) was purchased from Acros Organics BVBA (Geel, Belgium, <http://www.acros.com/>), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride

(EDC), N-hydroxysulfosuccinimide (sulfo-NHS), 2-(4-Morpholino) ethanesulfonic acid (MES), streptavidin, sucrose, Tween 20, bovine serum albumin (BSA), Hexadecyltrimethylammonium bromide (CTAB) and phosphate buffer saline (0.01 M, pH 7.4) were purchased from Sigma-Aldrich (St. Louis, MO, <http://www.sigmaaldrich.com/>). Glass fibers (GF000800), cellulose fiber (CFSP001700), nitrocellulose membranes (HF090MC100, HFB18004 and HFB24004) and laminated cards (HF000MC100) were purchased from Millipore (Billerica, MA, <http://www.merckmillipore.com/>). CA 19–9 protein, Mouse monoclonal CA 19–9 antibody 10-CA19A (Ab₁) and 10-CA19B (Ab₂) were purchased from Fitzgerald Industries International (Acton, MA, <https://www.fitzgerald-fii.com/>). Goat anti-mouse IgG (Ab₃) were purchased from ThermoFisher Scientific (Rockford, IL, <https://www.thermofisher.com/>).

All the chemicals used in this study were analytical reagent grade. Solutions were prepared with ultrapure (Z18 MΩ) water from Millipore Milli-Q water purification system (Billerica, MA, <http://www.merckmillipore.com/>).

Preparation of magnetized carbon nanotube (MCNTs)

Ten milligrams of multiwalled CNTs were treated with 4.8 mL H₂SO₄ and 1.6 mL HNO₃ under vigorous ultra-sonication for 6 h. The shortened CNTs was centrifuged 20 min at 6000 rpm min⁻¹, washed with water several times until the solution was neutral. During the washing steps, the shortened CNTs suspension was centrifuged 40 min at 14500 rpm min⁻¹. The collected pellet in the final washing step was suspended in 10 mL water for further use. MCNTs were prepared by coprecipitation method [30]. Briefly, 0.04054 g FeCl₃·6H₂O and 0.01491 g FeCl₂·4H₂O were added to 10 mL of shortened CNTs. The mixture was sonicated and stirred vigorously, and ammonia water was added dropwise under vigorous stirring till the pH value reached to 10, and kept stirring for 30 min. The brown MCNT were collected with an external magnet, washed with water for three times, and suspended in 10 mL water for further use.

Preparation of the MCNT-Ab₁ conjugates

The MCNT-Ab₁ conjugates were prepared according to the reported methods with slight modifications [31]. Two hundred and fifty microliters of MCNTs was mixed with 4.8 mg EDC and 2.7 mg sulfo-NHS in 0.5 mL MES buffer (0.1 M, pH 4.7). After shaking at room temperature for 15 min, the activated MCNTs were separated by an external magnet. Supernatant was discarded and pellet was re-suspended in phosphate buffer saline (0.01 M, pH 7.4). The above process was repeated three times to move away the extra reagents. Then added certain amount of Ab₁ to the activated MCNTs and incubated

the solution overnight at 4 °C. This mixture was washed by the same procedure as the activation process. Supernatant was discarded and the pellet was re-suspended in wash buffer (phosphate buffer saline +1% Tween). The above steps were repeated for 3 times, and then the pellet was re-suspended in 0.5 mL eluent buffer containing 20 mM Na₃PO₄·12H₂O, 5% BSA, 10% sucrose, and 0.25% Tween-20. The conjugate solution was stored at 4 °C.

Preparation of the lateral flow strip biosensor (LFSB)

The LFSB was made up of three components: sample application pad, nitrocellulose membrane and absorbent pad. Untreated glass fibers (GF000800) (23 mm × 30 cm) were used as the sample application pad. Ab₂ and Ab₃ were dispensed onto nitrocellulose membrane at 1 cm s⁻¹ to form the test and control zones with the aid of Biojet BJQ 3000 dispenser. The distance between the test and control zones was 4 mm. Then the membrane was dried at 37 °C for 1 h and kept it at 4 °C. Clamshell laminator was used to assemble three components on a plastic adhesive backing (60 mm × 30 cm) and every part overlapped 2 mm to ensure the solution migration along the strip. Finally, the strips with 3 mm width were cut by the Guillotin cutting module CM 4000 and stored at 4 °C before further use.

Assay procedure

Optimization of assay parameters in buffer

Assay parameters were first optimized in a pure buffer in the absence of blood. Sample solution with different concentrations of CA 19–9 were prepared with running buffer (phosphate buffer containing 1% BSA, 0.03% Tween, 1 mM CTAB). One hundred microliter of sample solution was mixed with 2.5 μL of Ab₁-MCNT conjugates. After gentle vortex, the mixture was applied to the sample pad of LFSB and the solution migrated toward absorption pad. Ten minutes later, another 60 μL of running buffer was added to wash the LFSB.

Detection of CA 19–9 in whole blood

Briefly, 20 μL of human blood spiked with a desired concentration of CA 19–9 was mixed with 80 μL of running buffer and 6 μL of MCNT-Ab₁ conjugates. The mixture was incubated 10 min under general shaking. After applying an external magnet 2 min, the MCNT-Ab₁-CA 19–9 complexes were separated from the whole blood, washed twice with phosphate buffer +1% Tween and re-suspended to 100 μL of running buffer. The MCNT-Ab₁-CA 19–9 complex solution was then applied the sample pad of LFSB. Ten minutes later, another 60 μL of running buffer was added to wash the LFSB. The test and control zones were evaluated visually within 20 min.

Quantitative measurements were obtained by reading the greyscale of the brown/black band on the test zone with the aid of a portable strip reader, which includes a digital camera and software.

Results and discussion

Principle of CA 19–9 detection in blood using MCNT and LFSB

MCNTs were prepared by coating Fe_3O_4 nanoparticles on the shortened multiwalled CNT surface via co-precipitation of ferric and ferrous ions within a dispersion of shorten multiwalled CNTs [30]. The shorten procedure of multiwalled CNTs under the mixture of HNO_3 and H_2SO_4 would introduce the carboxylic groups on the CNT surface, which increased the solubility of CNT and facilitated the immobilization of antibodies on the CNT surface. The morphologies of the shortened multiwalled CNTs and as-prepared MCNTs were investigated by scanning-electron microscope (SEM). One can see the shortened CNTs have a length of 3 to 5 μm (Fig. 1a). Magnetic nanoparticles were coated either on the surface or the ends of CNTs (Fig. 1c). Figure 1b shows the FTIR spectrum of the shortened CNT. The peaks of the shortened CNTs at 1800 and 1682 cm^{-1} indicated that the pretreatment in mixed acid generated carbonyl groups on the CNT surface.

Figure 1d presents the Raman spectrum of the shortened CNT (black line) and MCNT (red line). The dominant peak at $\sim 1350\text{ cm}^{-1}$ of CNT and MCNT can be attributed to the D band of CNTs, while another intense peak at $\sim 1576\text{ cm}^{-1}$ can be ascribed to the G band of CNTs. Three typical peaks of maghemite around 350, 500, and 700 cm^{-1} , matching well with the reported maghemite spectrum [32, 33]. The MCNTs were then used to conjugate with CA 19–9 antibody (Ab_1) by carbodiimide crosslinker chemistry via diimide-activated amidation between the carboxylic acid groups on the MCNT and amino groups on the antibody (Fig. 2a). The magnetized CNTs exhibited superparamagnetic property at room temperature and were separated from its solution within 40 s. The oxidized (shortened) CNTs wasn't separated from the suspension under the magnetic field. Figure 2b, c illustrates the principle of CA 19–9 detection in whole blood by using the as-prepared MCNT- Ab_1 and lateral flow strip biosensor (LFSB). First, the MCNT- Ab_1 conjugate is added to the blood in 1.5 mL centrifuge viral and incubated 10 min. After a magnetic separation and multiple washing steps, the formed MCNT- Ab_1 -CA 19–9 complexes are suspended in a running buffer and applied to the sample pad of LFSB. Mouse monoclonal CA 19–9 antibody 10-CA19B (Ab_2) and Goat anti-mouse CA 19–9 (Ab_3) were pre-immobilized on the nitrocellulose membrane to form the test zone and control zone, respectively. In the presence of CA 19–9 in the blood, the MCNT- Ab_1 -CA 19–9 complexes are captured by the Ab_2 on

Fig. 1 **a** Typical SEM image of shortened CNTs; **b** FTIR spectrum of shortened CNTs; **c** Typical SEM image of MCNT; **d** Raman spectrum of shortened CNTs (black line) and MCNTs (red line)

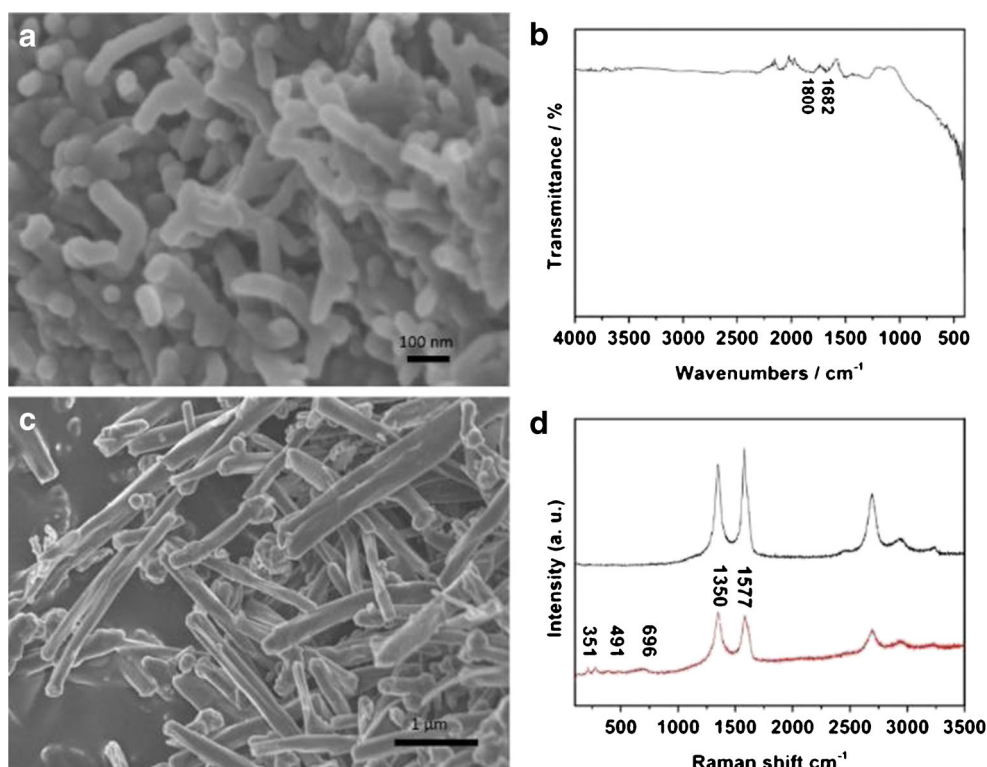
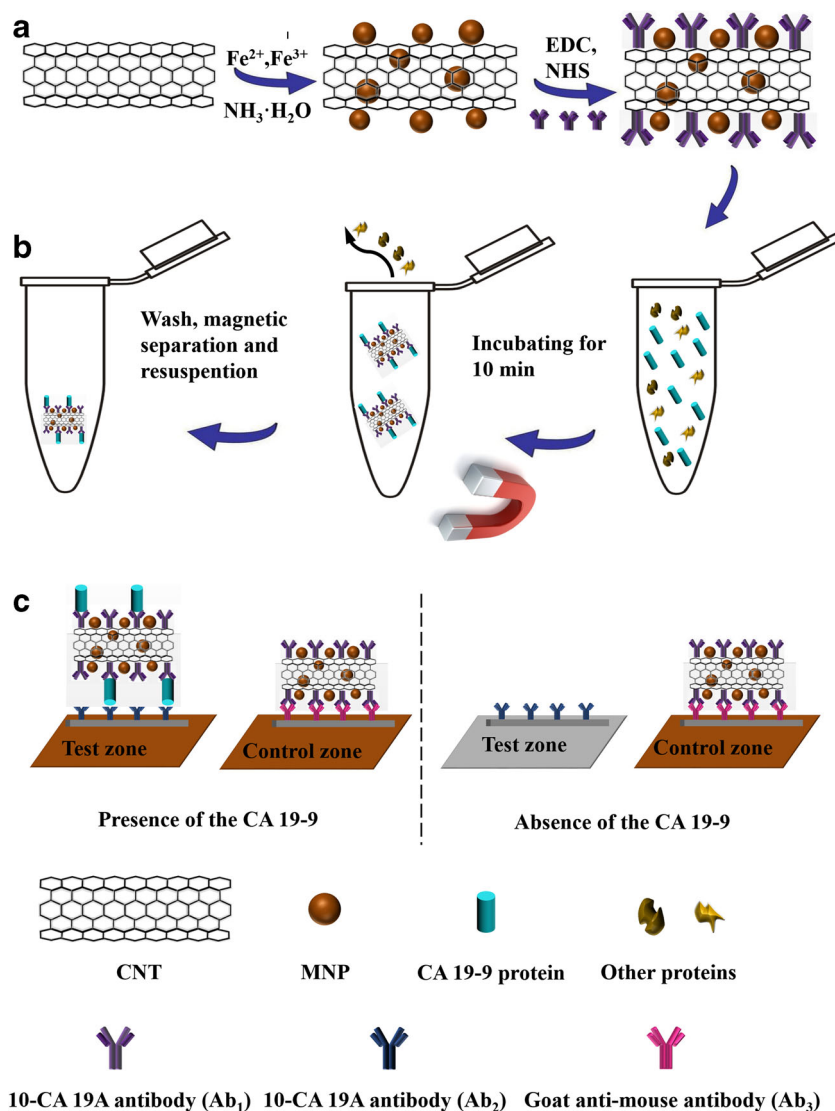


Fig. 2 **a** Schematic representation of preparation of MCNTs-Ab₁ conjugates, **b** capturing CA 19-9 in blood with MCNT-Ab₁, **c** measurement principle of the lateral flow strip biosensor in the absence and presence of CA 19-9



the test zone. The accumulation of the MCNTs on the test zone would form a distinct brown band, whose intensity was proportional to the concentration of CA 19-9 in the blood. The excess of MCNT-Ab₁ continued to migrate along the strip and captured by the second antibody (Ab₃) on the control zone to form another characteristic brown band. Qualitative analysis was realized by observing the color change of the test zone visually. Quantitative detection was obtained by reading the greyscale of the brown/black band on the test zone with the aid of a portable strip reader.

Optimization of assay parameters

Assay parameters of LFSB to detect CA-19-9 were first optimized in pure buffers in the absence of blood. Sample solutions containing different concentrations of CA 19-9 were prepared in the running buffer. The following parameters were

optimized: (a) running buffers; (b) capture antibody (Ab₂) amount on the test zone; (c) detection antibody amount (Ab₁) for preparing MCNT-Ab₁ conjugates; (d) the volume of MCNT-Ab₁ conjugates. Respective data and Figures (see Supplementary Material and Fig. S1) are given in the Electronic Supporting Information. The following experimental conditions were found to give best results: (a) A running buffer of phosphate buffer (0.01 M, pH 7.4) + 1% BSA + 0.03% Tween + 1 mM CTAB; (b) using 20 µg of Ab₁ to prepare MCNT-Ab₁ conjugates; (c) dispensing 1.0 mg mL⁻¹ of Ab₂ twice on the test zone; (d) using 2.5 µL of MCNT-Ab₁ conjugate per assay. Figure 3a presents the typical photo images (left) and corresponding optical responses (right) of the LFSBs with increasing CA 19-9 concentrations (2 to 200 U mL⁻¹) under the optimized experimental conditions. No band was observed on test zone of LFSB in the absence of CA 19-9 (control, 0 U mL⁻¹), indicating negligible nonspecific

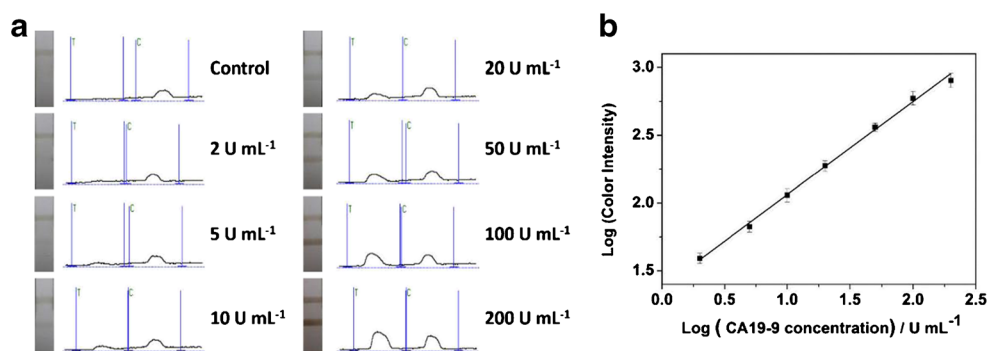


Fig. 3 **a** Typical photo images and corresponding optical responses of LFSBs with increasing CA 19–9 concentrations (2 to 200 U mL⁻¹); **b** calibration curve of the assay. Each data point represents the average

value from three different measurements; Assay time: 20 min; running buffer: phosphate buffer +1% BSA + 0.03% Tween +1 mM CTAB; dispensing time: two; the volume of MCNTs conjugate: 2.5 μ L

adsorption. The intensities of the test bands are increasing with the increase of CA 19–9 concentration. The test band can still be clearly observed at levels as low as 2 U mL⁻¹ of CA 19–9. Hence, sensitive visual detection is possible. Each sample were examined three times and the average intensity of each concentration was used to draw the calibration curve (Fig. 3b). Comparing the concentration vs color and log (concentration) vs log (color), the latter has better linear relationship. The resulting calibration plot of the logarithm of peak area versus logarithm of CA 19–9 concentration is linear in the range of 2 to 200 U mL⁻¹ with the detection limit of 1.75 U mL⁻¹ (S/N = 3). The method shows comparable detection limit to fluorescence and chemiluminescent immunoassay (Table S1). Although some methods in the table have shorter assay time and lower detection limit, they need sample-pretreatment and require expensive or complex readers, which are being utilized at the laboratory and cannot be used as a point-of-care detection method in resource limited areas. The selectivity of the assay was studied by testing a series of probable interferences including BSA, CEA, IgG, mammaglobin. As shown in Fig. 4, a high response is observed when 20 U mL⁻¹ CA 19–9 was tested, whereas the

negligible signals were obtained from other proteins with high concentrations, indicating the excellent specificity of the assay.

Analysis of CA 19–9 in whole blood

The blood sample was purchased from Golden West Biologicals, Inc. (Temecula, CA, www.goldenwestbio.com/) and the concentration of CA 19–9 in blood was tested with CA 19–9 ELISA kit (see details in ESI). The concentration of CA 19–9 in blood sample was 5.43 U mL⁻¹ (See Fig. S3). The blood samples containing different concentrations of CA 19–9 were prepared by spiking CA 19–9 standards in blood. The volume of MCNT-Ab₁ conjugates, the volume of blood used per assay, the incubation time of MCNT-Ab₁ in blood and magnetic separation time were optimized to obtain the best results (See Fig. S2). The following experimental conditions to give the best results: Twenty microliter of human blood spiked a desired concentration of CA 19–9 was mixed with 80 μ L of running buffer and 6 μ L of MCNT-Ab₁ conjugates. The mixture was incubated 10 min under general shaking. After applying an external magnet 2 min, the MCNT-Ab₁-

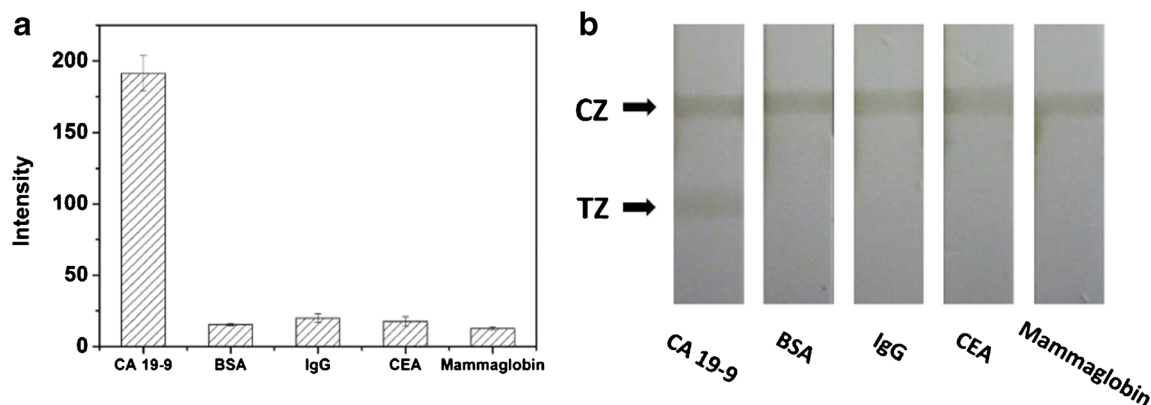


Fig. 4 Histogram of the LFSB responses and the corresponding photo images. Concentration of CA-19-9: 20 U mL⁻¹; concentration of BSA: 40 mg mL⁻¹, concentration of IgG: 80 mg mL⁻¹, concentration of CEA and Mammaglobin: 100 ng mL⁻¹. CZ: control zone; TZ: test zone

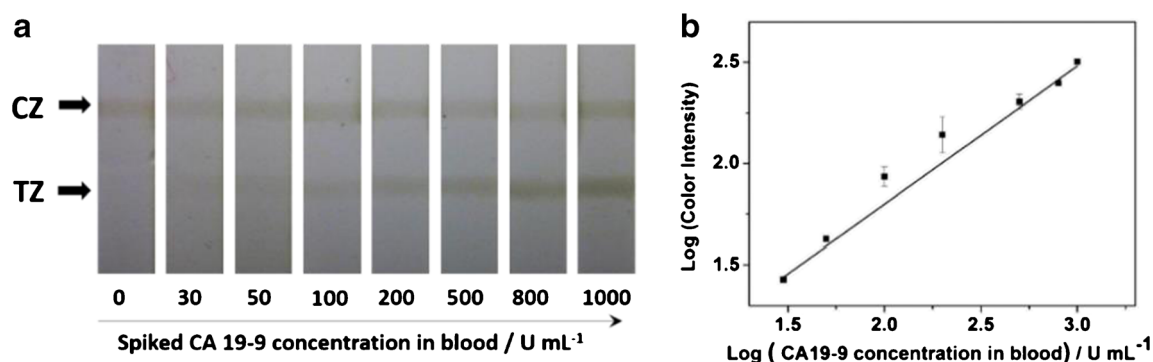


Fig. 5 **a** Typical photo images for the LFSB with an increasing CA 19–9 concentration in blood sample (0 to 1000 U mL⁻¹); **b** calibration curve. Each data point represents the average value from three different measurements

CA 19–9 complex were separated from the whole blood, washed twice with phosphate buffer +1% Tween. The use of MCNT-Ab1 to capture CA 19–9 in blood and washing steps reduced the matrix effect significantly. The complex was re-suspended to the optimized running buffer (phosphate buffer +1% BSA + 0.03% Tween +1 mM CTAB solution) before applying to the sample pad of the LFSB. Figure 5a shows a test band even at 30 U mL⁻¹ of CA19–9. No obvious red band was observed in whole blood without spiking CA 19–9. The intensities of the test bands of LFSB increased with the increase of spiked CA 19–9 in blood from 30 to 1000 U mL⁻¹ (Fig. 5b). The detection limit was 30 U mL⁻¹, which should be enough for diagnose use due to the cut off value for CA 19–9 is about 37 U mL⁻¹ in healthy people blood. The reproducibility of the assay was studied. Blood samples spiked 50 U mL⁻¹ CA 19–9 were tested six times that gave reproducible signals with a relative standard deviation (RSD) of 7.8% (See Fig. S3).

Conclusions

A rapid, low-cost and sensitive approach was developed to detect CA 19–9 in whole blood by using magnetized carbon nanotube (MCNT) and lateral flow strip biosensor. The MCNT composites combined the superpara-magnetism of Fe₃O₄ nanoparticles and the outstanding mechanical properties of CNTs which endowed MCNT-antibody conjugates with three functions: recognizing, separating, and visualizing. CA 19–9 in blood can be captured and separated by the MCNT-antibody conjugates and then detected on LFSB with a visual detection limit of 30 U mL⁻¹, below the cutoff value (37 U mL⁻¹) [14]. This holds promise for point-of-care diagnosis and clinical applications. To the best of our knowledge, this is the first time of the successful application of magnetized carbon nanotube and LFSB for the detection of CA 19–9 in blood avoiding sample purification and pre-treatment. Further work will aim to improve the detection limit of

assay and detect the large scale blood samples from healthy control and pancreatic cancer patients at different stages.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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